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ABSTRACT

Women with breast cancer are at increased risk for developing a second primary tumor in the lung. There may be a greater risk for women who receive radiotherapy and smoke. 110 cases with breast and lung cancer and 123 controls with breast cancer only were collected through the Swedish Cancer Registry in Stockholm, Sweden. Two pathologists jointly conducted a review to verify the registered primary status of the lung tumors. About 25% of lung tumors could not be confirmed as primary and these cases were excluded from data analysis. Four possible markers of susceptibility to lung cancer after breast cancer were examined: *p53* mutations, *p16* and *Ecad* methylation, and Estrogen Receptor Alpha (ERA). 81% of control breast tumors, 73% of case breast tumors, and 2% of case lung tumors were positive for ERA by immunohistochemistry. We found a decreased risk of lung cancer diagnosed less than ten years after breast cancer associated with strongly positive ERA expression in breast tumors (OR 0.08 CI 0.008-0.5). 2% of control breast tumors, 4% of case breast tumors, and 15% of case lung tumors were positive for *p16* promoter methylation. No breast tumors and 11% of case lung tumors were positive for *Ecad* promoter methylation. Methylation of *p16* or *Ecad* in lung tumors was associated with radiotherapy and ipsilateral lung tumors diagnosed greater than ten years after breast cancer (p=0.03). 4% of control breast tumors, 5% of case breast tumors, and 21% of case lung tumors were positive for *p53* mutations. Though limited by small sample size, this project will be a unique addition to the study of primary lung cancer after breast cancer. We found that as much as 25% of lung tumors registered as primary may actually be metastases, that ERA positive breast tumors are associated with a decreased risk of lung cancer diagnosed less than ten years after breast cancer, and that methylation of *p16* or *Ecad* is associated with lung tumors characteristic of radiation-induced cancer. Data from this study may provide information useful for defining subpopulations at increased risk for lung cancer after breast cancer, which may affect individual treatment decisions.

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Introduction

Radiotherapy has become a standard treatment for breast cancer; however, few studies have examined individual susceptibilities to risks from radiation exposure. Lung cancer following breast cancer has been associated with radiation exposure and this increased lung cancer risk has been shown to be even higher with tobacco exposure. These specific risks have not been explored at a molecular level, but previous studies have identified genetic traits that modify risks of breast or lung cancer and, in some cases, both cancers. Identification of molecular markers of radiation exposure may allow the distinction of groups of women susceptible to secondary lung cancer or multiple cancers and of women more significantly affected by smoking. Additionally, identification of molecular markers in breast and lung tumor tissue may suggest a common etiology for breast cancer and secondary lung cancer.

To study the risks associated with radiotherapy for breast cancer, we collected cases with breast cancer and secondary lung cancer and controls with breast cancer only from the Swedish Cancer Registry. We looked at breast and lung tumor tissue for mutations in *p53*, which is involved in a radiation response pathway and is strongly associated with DNA damage from smoking. Methylation of DNA is a key factor in the regulation of gene transcription and has been shown to contribute to carcinogenesis by blocking transcription of tumor suppressor genes. Based on this knowledge, we assessed the methylation of genes known to be involved in cancer progression. Finally, we conducted immunohistochemical studies to detect estrogen receptor alpha expression in breast and lung tumor tissue. Expression patterns may indicate a contribution of hormones to the etiology of these breast and lung tumors. This project may have significant clinical impact by providing additional information on risk to women choosing a breast cancer treatment. Additionally, this research may provide new data on the susceptibilities of women with multiple primary cancers and on hormone related gender differences in cancer risk.

Body

Background

Exposures

90% of lung cancer is related to tobacco. Smokers have a 10-20 fold increased risk of developing lung cancer compared to non-smokers, with risk related to the number of cigarettes smoked, depth of inhalation, and duration of cigarette use, among other factors. Reducing the number of cigarettes smoked per day decreases lung cancer risk, although low exposure, such as smoking a few cigarettes a day, still increases risk compared to non-smokers [1]. Smoking cessation lowers much of the risk of developing lung cancer, beginning 2-3 years after cessation and continuing for the next ten years. However, the risk of lung cancer in former smokers may remain elevated for as many as 30 years and will never be reduced to that of a never smoker [2]. Some studies have suggested that women are more susceptible to tobacco carcinogens than men, however, an understanding of the biological factors involved has not been achieved [3]. In patients with lung cancer, smoking has been associated with cancer recurrence, lung cancer-specific mortality and all-cause mortality. A recent study found that current smoking in lung cancer patients is an independent predictor of reduced survival, suggesting that direct biological pathways may mediate the effect of smoking on survival after lung cancer diagnosis. Smokers were also at greater risk of not receiving treatment [4]. Epidemiology and biochemical studies have provided strong evidence of smoking as a cause of lung cancer. Current research seeks to elucidate the complexity of the relationship between smoking and lung cancer, including the effect of individual genetic susceptibility. Genetic polymorphisms can affect lung cancer risk by influencing smoking behavior and nicotine addiction or by carcinogen metabolism.

Radiation has long been a suspect of cancer causation. In the first half of the 20th century, radiologists observed radiation-induced skin cancer, young women painting watches with radium were observed to have high rates of bone cancer, the radiographic contrast agent Thorotrast was found to increase liver cancer and leukemia rates, and leukemia excesses in radiologists were observed [5]. In the 1950's, studies of atomic bomb survivors began to appear and in the 1960's, the risk of excess cancer in underground miners was observed [6]. With these initial studies came a new understanding of the detrimental effects of radiation and the necessity of conducting studies of risk in different exposed populations.

Primary lung tumors following breast cancer radiotherapy are of particular interest because of the proximity of the lung to the areas of irradiation during therapy and because of the particularly poor survival rates of lung cancer patients. A study utilizing SEER data found an increased risk of ipsilateral lung cancer in women ten years after radiotherapy for breast cancer. This study was limited by lack of detailed radiotherapy data that may have been misclassified in the registry and by a lack of significance of the observed increase in risk [7]. A group using the Connecticut Tumor Registry, limited to women who developed a second malignancy between 1986 and 1989, found a three-fold increase in risk of lung cancer more than ten years after radiotherapy for breast cancer. This study analyzed only 89 cases and had wide confidence intervals. The study was also limited by the controls used, which were women with second cancers not related to an exposure, and its reliance entirely on registry data [8]. Another Connecticut Tumor Registry study found that women who survived at least ten years after diagnosis of breast cancer had an elevated risk of lung cancer that continued to increase with time after diagnosis [9]. Access to medical records allowed researchers in this study to determine radiation dose, and an excess relative risk of 0.2 per Gy to the affected lung was observed. While this study was strengthened by access to dose information and greater than ten years of follow-up, it was limited by sparse smoking data.

In a study comparing breast cancer patients who received surgery and radiotherapy with breast cancer patients who did not receive radiotherapy, an increased risk for lung cancer was observed ten or more years after treatment [10]. This study excluded all second cancers diagnosed at the same site within one year of the original breast tumor, reducing the chance of including spread from the first tumor as a new tumor, but only excluded cancers at different sites if they were diagnosed on the same day as the original breast cancer [10]. A case control study of women with second malignancies after breast cancer treatment found an increasing risk of cancer with increasing dose of radiotherapy and an increased risk of lung cancer associated with radiotherapy [11]. A study of early-stage breast cancer patients treated with lumpectomy and radiation therapy found a significant increase in lung cancer after five years, with a higher risk in women diagnosed with breast cancer younger than 50 years old. This study had a follow-up of only eight years and risk may have been underestimated due to conservative classification of lung tumors as primary. Also, more than 50% of patients received supraclavicular irradiation, which may account for the detected risk in this study compared to others [12]. A large SEER study, including over 270,000 subjects, found that women who had postmastectomy radiation had an increased risk of lung cancer at 10-14 and 15+ years after radiotherapy, but women who received post-lumpectomy radiation had no increased risk up to 14 years after radiotherapy. This suggested that the highest risk of secondary lung cancer was in patients who were treated in the 1970's and 1980's, when higher doses of radiation were given after surgery. However, no dose information was available, nor was smoking data, as only database information was used [13].

None of the previously described studies were able to perform any histological confirmation of lung tumor diagnosis, which could lead to misclassification of metastatic lung tumors as primary and cause an overestimation of risk. Pathology medical record review was conducted in two randomized trials that were part of the National Surgical Adjuvant Breast and Bowel Project, where patients were randomized in

one trial to radical mastectomy versus total mastectomy and radiotherapy (1971-1974) and in another trial to total mastectomy versus lumpectomy and breast irradiation (1976-1984) [14]. Researchers on this study enlisted two physicians to review medical records of cases to validate primary diagnoses. Lung tumors were considered primary if they had a different histology from the original breast cancer or if the diagnosis was adenocarcinoma and the radiographic findings were compatible with a primary tumor. Lung tumors were considered probable primaries if they had a solitary lung nodule and were an ambiguous adenocarcinoma. Tumors were classified as metastatic if they had multiple lung nodules or pleural effusion with histology similar to the original breast tumor [14]. Even with access to current, complete medical records, there were still cases that could not be definitively categorized, emphasizing the challenge of verifying second primary tumors in the lung in registry studies. In the first trial, there was an increased risk of lung cancer in women who received post-mastectomy radiation and a significant increase in ipsilateral lung cancer, which was not significant when probable primary tumors were added to the analysis. In the second trial, there was no increased risk of any lung tumor in patients who had received radiotherapy, presumably due to the decrease in radiation field used in the later years [14].

Several studies of Hodgkin's disease patients have found a significant increase in lung cancer risk with smoking and radiotherapy and a greater risk for smokers with longer pack-year histories [15-17]. A study of head and neck squamous cancers found better survival in nonsmokers or very light smokers compared to light, moderate, and heavy smokers [18]. In a study comparing breast cancer patients receiving lumpectomy and radiotherapy versus mastectomy without radiation, researchers found a 20% risk of developing a second malignancy in15 years for smokers, compared to 16% for nonsmokers. A 15-year risk of developing lung cancer was observed at 0.26% for nonsmokers, 4.7% for patients with a smoking history before radiation, and 6% for patients who continued to smoke at the time of radiation. Researchers were able to review pathology reports to confirm second cancer diagnosis, but did not review original materials [19]. A study of patients from the Connecticut Tumor registry found a multiplicative effect of smoking and radiation on lung cancer risk, with a 32.7 relative risk for women exposed to cigarette smoking and radiotherapy for breast cancer. This study had smoking data on 77% of subjects, but only 89 cases and wide confidence intervals in the analysis. This study also relied exclusively on registry data and, in order to get more smoking data, used cancer patients with second cancer not related to exposure as controls [8].

A retrospective hospital-based study of women with breast cancer found an odds ratio of 9.0 for lung cancer in women who received radiotherapy and smoked, but found no increased risk for radiotherapy alone [20] . This study reported the most comprehensive pathology review to establish lung tumors as primary, enlisting a pathologist to review reports, tissues, or documented correspondence from other institutions. Of the 380 potential cases in the retrospective analysis, 29 were excluded due to misclassification of breast or lung tissue as from another organ and 16 were excluded due to the inability to rule out the lung tumor as a metastasis. However, the study was still limited by possible misclassification of tumors, despite the pathology review, because most data came from medical records. They also had to adjust for age in the analysis because good age matching between cases and controls could not be achieved. In our study using the Swedish Cancer Registry, a review of breast cancer patients with secondary lung cancer found an increased risk of lung cancer related to radiotherapy, but only in smokers and the confidence interval included one [21]. This study benefited from access to radiation dose information and smoking data from medical records or next-of-kin interviews, but was limited by a lack of pathological confirmation of lung tumor status.

Markers
p53

The *p53* gene encodes a protein that does not function correctly in most human cancers. In about half of tumors, *p53* is inactivated directly by mutation, but can also be inactivated by binding with viral proteins or due to alterations in genes whose products interact or communicate with *p53*. Most mutations result in the inability of the protein to activate transcription [22]. However, recent studies have shown that some p53 mutants acquire functions that wild type p53 does not have, such as enhancement of carcinogenicity and invasiveness.

p53 is mutated in 20-40% of breast cancers. The IARC database contains 2,209 *p53* mutations in breast cancer, with 67% missense mutations, 21% G to A transitions, and 22% G to A transitions at CpG sites. Hotspot codons with frequent mutations include 175, 220, 245, 248, 273, and 282. The pattern and codon distribution of *p53* mutations in breast tumors is similar to all cancers, with the exception of a lower number of G to T transversions and a hotspot at codon 220 [23]. Evidence of *p53* mutations before the presence of invasive cancer suggests that *p53* mutation may be an early event in breast cancer. The increasing frequency of mutations through breast cancer progression may lead to different reported mutation rates if studies use populations at different stages of cancer [24]. Most information on *p53* mutation rates in breast cancer is in smokers. Two studies have investigated p53 in the breast tumors of smokers, one with IHC and one with mutation analysis. In the IHC study, 378 breast tumors were stained for p53 protein expression. 44% of tumors were positive and p53 expression was more common in ER negative tumors. They found that positive p53 status was associated with cigarette smoking, especially in ER positive tumors [25]. The study using mutation analysis found a higher prevalence of *p53* mutations in the breast tumors of smokers compared to never-smokers; they also were more likely to have *p53* transversions and G to T transversions [26]. They suggested that exposure to cigarette smoke may have genotoxic effects in breast tissue and that it may modify the prevalence and spectrum of *p53* mutations in breast cancer. However, much more evidence must be generated before any concrete conclusions about *p53* and smoking in breast cancer can be made.

The *p53* gene is mutated in about 50% of lung cancers. Among the 2,372 mutations recorded for lung cancer in the IARC database, 75% of mutations are missense and GC>TA is the most common type of mutation. Common codons with mutations are 157, 158, 175, 245, 248, 249, 273, and 282 [23]. Tobacco smoke has been definitively established as the major cause of lung cancer and the presence of a pattern of *p53* mutations has strongly supported the causal relationship. As 90% of lung cancers are related to smoking, studies of *p53* mutations are largely done in smokers, though as larger studies are conducted more information on nonsmokers is becoming available. The spectrum of *p53* mutations in lung cancer is different from other cancers, and only hepatocellular carcinomas also have a significant presence of G to T transversions. PAHs are a well-studied component of tobacco smoke and have been demonstrated to be carcinogenic to lung tissue. DNA damage from PAHs appears to be nonrandom, as several codons have been consistently associated with PAH adducts. These codons, 157, 158, 245, 248, and 273, are also sites of high *p53* mutation frequency in lung studies [27]. Differences in *p53* mutation spectra related to gender have been suggested based on hormones, behavior, or different susceptibility to carcinogens, but strong evidence for this does not exist. There may, however, be differences between female smokers and never smokers that relate to these conditions [28;29].

p53 plays a well known role in cellular pathways responding to DNA damage by radiation. Studies have shown that p53 mediates radiation-induced apoptosis and cell-cycle arrest to maintain genome stability after cellular insult. Cells lacking p53 can become radioresistant and many clinical studies have demonstrated reduced local tumor control and survival with mutated *p53* [30]. Exposure to radiation can lead to different types of DNA and chromosome damage, but researchers not yet found a p53 mutation

spectrum unique to radiation exposure, as has been identified for tobacco exposure. Studies of residential radon exposure have found an increase in mutations, especially among nonsmokers [31]. A study of normal bronchial epithelial cells found that high linear energy transfer (LET) radiation induced G to A transitions at codon 249 and C to A transversions at codon 250 [32]. While a *p53* mutation spectrum for radiation exposure is not as clear as for cigarette smoke, some evidence of a difference in the spectra and a possible mutation typical of radon exposure support further investigations.

Estrogen Receptor

Estrogen has long been known as an integral part of normal mammary development and as influential in breast tumorigenesis. The main path of activity for estrogen is through the regulation of expression of target genes that have estrogen-responsive elements in their promoters. Estrogen Receptor Alpha (ERA) binds ligands, undergoes conformational changes that result in dissociation of heat shock proteins, forms a dimer, and modulates transcription of estrogen responsive genes through interaction with coactivators or corepressors. The estrogen-regulated proteins resulting from the modulation of different transcriptional pathways by ERA function as growth factors and underlie the mitogenic action of estrogen. ERA can also regulate gene expression through protein-protein interactions in other transcriptional pathways [33]. A third mechanism of ERA activity is through nongenomic effects, occurring outside the nucleus through membrane bound ERA and other signal-transduction pathways [34].

The percentage of ERA positive cells is typically low in normal mammary glands, but increases in benign proliferative disease and low-grade ductal carcinoma in situ. An inverse relationship exists between ERA expression and proliferation in normal mammary epithelial cells, suggesting that estrogen controls proliferation by secretion of growth factors. This may allow attenuation of the sensitivity of normal mammary epithelium and ensure that proliferation occurs only when needed. Deregulation of the relationship between ERA expression and proliferation occurs in early stages of breast tumor development and 70% of invasive breast tumors express ERA [35]. Positive ERA status correlates with favorable prognostic factors after diagnosis of breast cancer, including lower rate of proliferation and histologic evidence of tumor differentiation. Lack of ERA expression in breast tumors is correlated with more malignant disease and poor prognosis [33]. Absence of ERA can result from hypermethylation of the promoter region of the gene, which may provide the cell with a growth advantage. ERA negative cells are no longer being regulated by estrogen in their growth and are not receptive to regression through reduction of estrogen levels or alteration of receptor activity [36].

Data on ERA expression in the lung is inconsistent in current literature. Variability could be due to study sample size, antibody choice, antigen retrieval techniques, or quantification of positive expression. A recent immunohistochemistry study in lung adenocarcinomas found 18% of samples positive for ERA using the monoclonal ID5 antibody, the most common clinically used clone. However, previous studies using this clone have detected anywhere from 0% to 67% of samples positive [37]. A comparative immunohistochemistry study in pulmonary adenocarcinoma used the ID5 clone and the monoclonal 6F11 clone to stain 45 tumors and found 66% positive for ERA expression using the 6F11 clone and 0% positive using the ID5 clone [38]. In contrast, a study of non-small cell lung cancer using a similar approach in 32 tumors found only one positive sample with each clone, one adenocarcinoma and one squamous cell carcinoma [39]. A study of non-small cell carcinoma, mostly adenocarcinoma, comparing the monoclonal ID5 clone to a polyclonal antibody found 0% staining with ID5 and 73% staining with the polyclonal antibody. These antibodies bind to different regions of the ERA protein, suggesting the presence of an ERA variant in lung tumors that may be a more significant source of ERA than the complete ERA protein [40]. ERA may have prognostic value in non-small cell lung cancer, as observed in an immunohistochemical study that found ERA associated with poorer prognosis for early stage tumors

and overall survival [40]. Analysis of ERA by another immunohistochemistry study of non-small cell lung cancer suggested that ERA is associated with histologic grade, poor prognosis, and smoking [41].

Methylation

The most common epigenetic modification in humans is DNA methylation. Methyl groups are added to the carbon five position of cytosines within the dinucleotide CpG. About 4-6% of all cytosines are methylated in normal DNA. CpG dinucleotides occur in clusters known as CpG islands and are usually unmethylated in normal tissues. CpG islands frequently occur at the 5' end of a gene and when they remain unmethylated, in conjunction with other transcription factors, allow gene transcription. Normal methylation of CpG islands occurs in specific instances: imprinted genes, X-chromosome genes in women, germline-specific genes, and tissue-specific genes [42]. In cancer cells, the balance of methylation is disturbed, resulting in global genomic hypomethylation and gene-specific hypermethylation.

Promoter hypermethylation has been detected in the genes of many pathways: cell cycle regulation, DNA repair, apoptosis, drug resistance, detoxification, differentiation, angiogenesis, and metastasis. Some genes are commonly methylated in many cancers, while some are methylated only in specific cancers. Many tumors will show hypermethylation in multiple genes [43]. Methylation can facilitate mutagenesis and genomic instability by silencing DNA repair or cell cycle regulation genes and can influence carcinogenesis by affecting expression of mutated genes [44]. Methylation profiles associated with certain types of cancer could be used to identify cancer and, when combined with techniques using biological fluids and biopsies for detection, could provide a valuable clinical tool for diagnosis. The presence of methylation patterns in precursor lesions that are similar to the patterns in sporadic cases may be used in early diagnosis of cancers [43].

p16 is a cell-cycle regulatory protein that is involved in tumor suppression through the Rb pathway. Disruption of p16 activity can result in uncontrolled cell proliferation [45]. Loss of p16 expression is a common feature of non-small cell lung cancer and can occur through mutation, deletions, and hypermethylation of the promoter region. In a study of squamous cell carcinoma of the lung, 44% of samples negative for p16 expression were hypermethylated at the *p16* promoter, however the study had only 13 samples [46]. In a study of 29 non-small cell lung cancers, all tumors exhibiting methylation had abnormal p16 expression by immunohistochemistry (IHC) [47]. A study of human mammary epithelial cell lines found that *p16* methylation was associated with *p16* inactivation and suggested that specific sites of methylation are more important than the total number of methylation sites [48]. In a breast cancer study, *p16* promoter methylation was found to be the major mechanism of inactivation for the gene, with hemizygous deletion as the second most common cause [45]. These studies provide evidence that *p16* inactivation is often caused by methylation.

Studies of breast carcinoma cell lines have found frequent hypermethylation of *p16*, however, methylation of breast tumors is not as common [49-51]. In a study of 54 sporadic breast cancers, 18% of tumors were hypermethylated at the *p16* promoter [52]. A study of sporadic and familial breast tumors found similar rates of *p16* hypermethylation in both sets of tumors, with 15% for sporadic and 18% for BRCA1 families [53]. A study of 100 breast carcinomas found 19% of samples had *p16* hypermethylation and that this methylation was correlated with poor prognosis [54]. Hypermethylation of *p16* has been more extensively studied in lung cancer. In studies looking at DNA methylation profiles in non-small cell lung cancers, *p16* was hypermethylated in 30-67% of tumors [47;55-60]. About 10% of small cell cancers are hypermethylated at *p16* [55]. Methylation of *p16* was found in 47% of non-small cell lung tumors and was associated with squamous cell carcinoma and patients with distant metastases in a study looking at clinicopathological characteristics [61]. Some studies have investigated the use of *p16* methylation as a

prognostic factor, suggesting that *p16* methylation is associated with poor prognosis alone or in combination with unmethylated *E-cadherin* (*Ecad*) [62-65]. However, conflicting studies have been published, so more studies are required to determine the utility of *p16* methylation as a prognostic marker [59;66;67].

E-cadherin (Ecad) is as transmembrane glycoprotein important in maintaining cell-cell adhesion in epithelial tissues [68]. Mutations in *Ecad* have frequently been absent in immunohistochemical studies that have observed reduced or absent expression of the gene in breast carcinomas, supporting methylation as a mechanism for loss [69]. Reduced Ecad expression has been found in 50% of invasive ductal carcinomas and almost 100% of infiltrating lobular carcinoma. A study of lobular carcinoma found hypermethylation of *Ecad* in 41% of tumors and reduced expression by IHC in all but one methylation positive tumor. A high frequency of LOH was observed, but there was an inverse relationship between loss of heterozygosity and methylation, suggesting methylation as an alternative path to gene silencing in lobular breast carcinoma [70]. In a recent study of 71 ductal breast carcinomas, about 75% were methylated at *Ecad* and 65% had reduced Ecad expression in combination with methylation [69]

Methylation of *Ecad* does not occur as frequently in lung tumors as in breast tumors, however, *Ecad* does seem to have a role in lung cancer progression. In three studies of Ecad in non-small cell lung carcinoma, reduced expression of Ecad was correlated with decreased differentiation. One study looked specifically at squamous cell carcinomas of the lung and found that 67% had decreased or absent expression of Ecad, while a second found decreased or absent expression in 73% of squamous cell tumors, 37% of adenocarcinomas, and 60% of bronchioalveolar carcinomas. Decreased Ecad expression was also correlated with increased stage and lymph node metastases [71-73]. In a study of non-small cell lung cancer and corresponding non-neoplastic lung tissue, *Ecad* was methylated in 29% of tumors and 15% of non-neoplastic tissue [56]. In a study of multiple histologies, *Ecad* was methylated in about 30% of adenocarcinomas and 30% of squamous cell carcinomas and about 60% of small cell tumors [55].

Exposure to radiation or tobacco that results in DNA damage could lead to disrupted replication or loss of normal transcription, which have been proposed as possible mechanisms of aberrant hypermethylation [74]. In studies of non-small cell lung cancer, researchers found that smoking was associated with *p16* promoter hypermethylation, with loss of protein expression, and with histological type [56;75]. Methylation of *p16* has been positively associated with pack-years smoked, duration of smoking, and negatively with time since quitting [76;77]. In the past, molecular mechanisms leading to DNA damage after radiation have been believed to work primarily through large deletions and point mutations. However, recent work suggests that elevated cancer risk from radiation may in part be mediated by promoter methylation [78]. In a study of human keratinocytes, cells treated with low dose irradiation or with medium from irradiated cells demonstrated deregulation of DNA methylation, predominantly hypermethylation of CpG dinucleotides, up to 20 passages after exposure.

A study of Chernobyl clean-up workers did not find *p16* hypermethylation in bronchial epithelia associated with radiation exposure, only with smokers compared to nonsmokers [79]. However, in a renal cell carcinoma study of residents from contaminated areas near Chernobyl, *p16* methylation was present in a higher percentage of patients from contaminated areas than in controls from uncontaminated areas. Oxidative stress resulting from prolonged irradiation was present, as evidenced by an increase in expression of COX2 [80]. In a study of former uranium miners, *p16* methylation was not associated with radon exposure, potentially because 75% of the tumors from the miners were peripheral and radon particles are typically deposited in the central lung. However, in the total study population of smokers, nonsmokers, and miners, *p16* methylation was associated with central tumors [81]. One of the most supportive studies for the role of radiation in promoter methylation was done in Mayak workers in Russia who were exposed to plutonium. *p16* hypermethylation was increased significantly in workers compared

to non-worker controls and a dose response was seen after stratification for level of plutonium exposure. The number of genes methylated also increased with plutonium dose [78]. These studies suggest that radiation induced DNA damage could be affected through epigenetic modifications, in addition to the more commonly explored pathways.

Results

Clinical Characteristics: Women who developed breast cancer and subsequently developed lung cancer were cases and women with breast cancer who never had lung cancer were controls. 180 cases with breast cancer diagnosed between 1958-2000 and lung cancer diagnosed at least one year after the breast cancer were identified through the Swedish Cancer Registry. The clinical characteristics are shown in Table 1. 70 cases were excluded for lack of tissue, resulting in a total of 110 cases. Cases were matched to 123 controls on age at breast cancer diagnosis, decade of diagnosis, and for the number of years survived since the diagnosis of the breast cancer. There were 3 cases and 18 controls with tumor tissue analyzed but no clinical data available.

Mean age at breast cancer diagnosis for cases and controls was 56 and 57 and mean age at lung cancer diagnosis for cases was 68. Availability of information on menopausal status in medical records was inconsistent, so age greater or less than 50 was used as a surrogate for menopausal status. 33% of controls and 35% of cases were premenopausal at the time of breast cancer diagnosis. Mean time to diagnosis of lung cancer after breast cancer in cases was 13 years. 48% of lung cancers were ipsilateral (occurring on the same side as the breast cancer), 38% were contralateral (occurring on the opposite side of the breast cancer), and 14% were of unrecorded location. 68 (62%) cases were smokers, of which 9 (13%) were identified through next-of-kin interviews. 23 (19%) controls were smokers, of which 4 (17%) were identified through next of kin interviews. Of the samples with smoking information from medical records and next-of-kin interviews, 88% of controls and 91% of cases were concordant (Table 2A). 60% of cases and 48% of controls received radiotherapy to treat breast cancer. Crude odds ratios for risk of lung cancer with exposure to radiotherapy (OR 1.6, CI 1.2-3.0) and smoking (OR 7.2, CI 3.4-15.0) indicate increased risk with either exposure (Table 2B).

Table 1. Description of clinical characteristics of study cases and controls.

Clinical Characteristics	Control		All cases[2]		Cases with score 4 or 5[3]		Cases with score 3[4]		Cases with score 1 or 2[5]	
Mean age at breast cancer diagnosis	57		57		56		62		58	
Age at breast cancer diagnosis[1]	n=123	%	n=110	%	n=83	%	n=11	%	n=16	%
50 or younger	41	33	38	35	29	35	3	27	6	38
51 or older	64	52	69	63	52	63	8	73	9	56
no data	18	15	3	3	2	2	0	0	1	6
Year of breast cancer diagnosis	n=105	%	n=107	%	n=81	%	n=11	%	n=15	%
1958-1969	36	35	37	35	26	32	4	36	7	47
1970-1979	34	33	35	33	24	30	6	55	5	33
1980-1989	27	26	28	26	24	30	1	9	3	20
1990-1997	7	7	7	7	7	9	0	0	0	0
Mean age at lung cancer diagnosis			68		68		75		65	
Mean years to diagnosis of lung cancer			13		13		13		11	
Latency			n=107	%	n=81	%	n=11	%	n=15	%
<3 years			7	6	6	7	0	0	1	7
3-5 years			14	12	10	12	0	0	4	27
6-10 years			29	25	22	27	5	46	2	13
11-20 years			35	30	25	31	4	36	6	40
21-30 years			18	15	15	19	1	9	2	13
>30 years			4	3	3	4	1	9	0	0
Lung cancer location			n=110	%	n=83	%	n=11	%	n=16	%
ipsilateral			53	48	45	54	3	27	5	31
contralateral			42	38	28	34	7	64	7	44
unknown			15	14	10	12	1	9	4	25
Smoking	n=123	%	n=110	%	n=83	%	n=11	%	n=16	%
yes	23	19	68	62	55	66	8	73	5	31
no	54	44	30	27	18	22	3	27	9	56
no information	46	37	12	11	10	12	0	0	2	13
Radiotherapy	n=123		n=110		n=83		n=11	%	n=16	%
yes	59	48	66	60	54	65	8	73	9	56
no	46	37	41	37	27	33	3	27	6	38
no information	18	15	3	3	2	2	0	0	1	6

1- Age 50 used as a surrogate for menopause due to lack of information in medical records. 2- All cases with tissue available identified in the Swedish Cancer Registry. 3- Only cases with scores of 4 or 5 in the pathology review, classified as primary. 4- Only cases with a score of 3 in the pathology review, classified as undetermined. 5- Only cases with scores of 1 or 2 in the pathology review, classified as metastatic.

Table 2. A. Concordance of smoking data between breast cancer records and next-of-kin interviews for cases and controls with information from both sources. B. Crude odds ratio analysis for risk of lung cancer with exposure to smoking or radiotherapy for breast cancer.

A.

	Smoking data from breast cancer records			
	controls		cases	
Smoking data from next-of-kin interview	yes	no	yes	no
yes	9	2	29	3
no	1	13	1	11
	p=0.001		p=0.001	

B.

	Crude odds ratio	95% CI
Smoking	7.2	3.4-15.0
Radiotherapy	1.6	1.2-3.0

Pathology verification of cancer diagnosis: Cases included in the study were recorded in the Swedish Cancer Registry as having primary lung cancer. In an effort to ensure validity of the study, a pathologist at GU conducted a review of the cases. This review utilized H&E stains from the breast and lung tumors of each case, examined in pairs to compare tumor morphology. After the review, all breast tumors from cases and controls were confirmed as primary breast tumors. 49 cases were deemed questionable as having primary lung tumors and an additional review was conducted. Two pathologists collaborated to confirm histology: one with expertise in breast cancer and one in lung cancer. The joint analysis was conducted with a multi-head microscope, examining H&E, ERA, and TTF-1 stains. 81% of control breast tumors, 74% of case breast tumors, and 10% of case lung tumors were positive for ERA. An algorithm was developed to achieve a consensus on the assignment of a score from 1-5 for the lung tumors, ranging from a score of one for definitely a breast cancer metastasis to a score of 5 for definitely a primary lung tumor. The algorithm classified lung tumors based on morphology, TTF-1 lung tumor staining, and ERA status in matched breast and lung tumors. Positive TTF-1 staining was considered indicative of primary lung tumor status, while an ERA positive lung tumor with an ERA positive matched breast tumor was considered a possible metastasis. 45% of these reviewed cases were included in the study (scores of 4 or 5) and 55% were excluded as undetermined (score of 3) or metastatic (score of 2 or 1) (Table 3). Cases confirmed as primary after the first review were automatically assigned a score of 5 for the data analysis. TTF-1 data was sparse due to quality control problems, however, tumors in both the undetermined and metastatic categories that did not have TTF-1 staining data could move to the primary category if a positive stain were provided in the future.

83 (75%) of the original study set of 110 cases were considered to have primary lung tumors and were included in the analysis. Data was generated for undetermined or metastatic cases because of the possibility that gathering further data in the future (namely TTF-1 staining) might allow the inclusion of some samples. There may also be an opportunity to investigate biomarkers in women with breast cancer that metastasizes to the lung. Undetermined or metastatic cases were not included in the statistical analysis. Among the final 83 cases with confirmed lung cancer, 29% of primary lung tumors were squamous cell carcinoma, 20% were adenocarcinoma, 19% were small cell carcinoma, 14% were mixed adenocarcinoma/squamous cell carcinoma, 11% were bronchioloalveolar, 2% were mucinous adenocarcinoma, and 3% were large cell carcinoma. 8 (73%) of the undetermined lung tumors and 13 (81%) of metastases were adenocarcinoma (Table 3). All squamous and small cell lung cancers were considered primary, while half of adenocarcinomas were undetermined or metastatic (Figure 1).

Figure 2 shows the distribution of cases by lung tumor pathology review score within decades of breast cancer diagnosis. This graph demonstrates that similar percentages of cases were excluded from each

decade when the analysis was done, indicating very little bias against any particular decade of breast cancer diagnosis. Figure 3 shows the distribution of cases by lung tumor pathology review score within decade of lung tumor diagnosis. This demonstrates that similar percentages of cases were excluded from each decade when analysis was done, indicating very little bias against any particular decade of lung cancer diagnosis. Figure 4 shows the distribution of cases by lung tumor pathology review score across decades of lung tumor diagnosis. This graph shows that the percentage of primary lung tumors diagnosed increased with each decade, suggesting that diagnosis became more accurate with time. Figure 5 shows the distribution of latency (time between diagnosis of breast and lung cancers) of lung tumors in cases for each of the pathology review score categories, demonstrating that there was no significant bias due to exclusion of tumors from analysis based on latency. Latency was not used as criteria for classification of tumors in the pathology review, even though longer latency would suggest a primary tumor status. Over 50% of undetermined tumors had a latency of longer than 10 years, while almost 50% of the metastatic tumors had a latency of less than 10 years. Assay data is presented for the categories of “All Cases”, which includes cases with any pathology review score, “Scores 4 and 5”, “Score 3”, and “Scores 1 and 2”.

Table 3. The histology of case lung tumors in categories as determined by the pathology review score. Scores 4 and 5 were primary, score 3 was undetermined, and scores 1 and 2 were probable metastases.

Final Case Pathology Designation		
Primary (scores 4,5)		
	n	%
Adenocarcinoma	17	20
Squamous cell carcinoma	23	28
Adeno/squamous cell carcinoma	12	14
Bronchioloalveolar carcinoma	9	11
Squamous cell carcinoma/sarcomatoid	1	1
Mucinous adenocarcinoma	2	2
Large cell	3	4
Small cell	16	19
Total	83	
Undetermined (scores 3)		
	n	%
Adenocarcinoma	8	73
Squamous cell carcinoma	0	0
Adeno/squamous cell carcinoma	3	27
Bronchioloalveolar carcinoma	0	0
Squamous cell carcinoma/sarcomatoid	0	0
Mucinous adenocarcinoma	0	0
Large cell	0	0
Small cell	0	0
Total	11	
Metastases (scores 1,2)		
	n	%
Adenocarcinoma	13	81
Squamous cell carcinoma	0	0
Adeno/squamous cell carcinoma	0	0
Bronchioloalveolar	1	6
Squamous cell carcinoma/sarcomatoid	0	0
Mucinous adenocarcinoma	2	13
Large cell	0	0
Small cell	0	0
Total	16	

Figure 1. Distribution of pathology review score groups within histologic type of case lung tumors. Squamous and small cell carcinomas were only in the primary group, while adenocarcinomas were included all groups. Most probable metastases were adenocarcinomas.

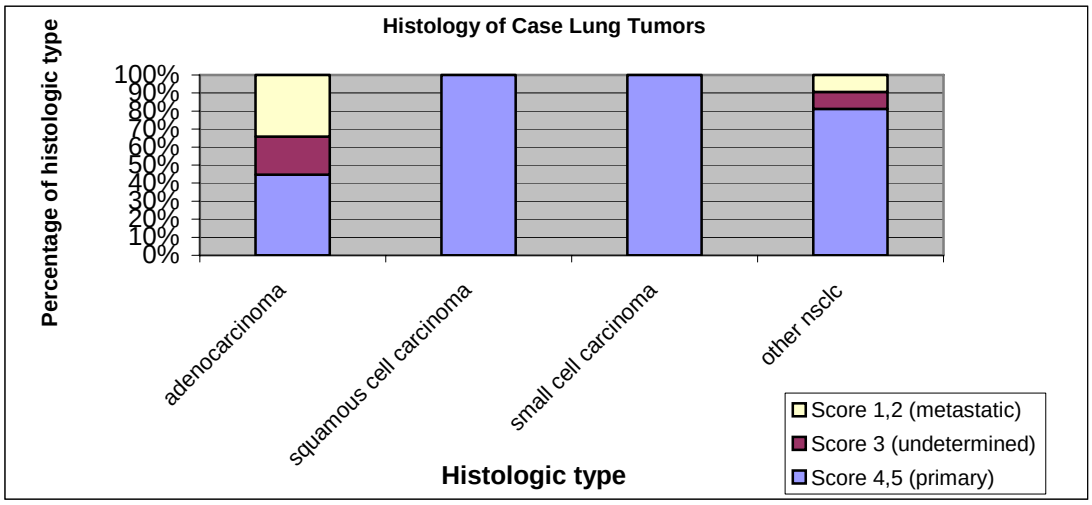


Figure 2. Distribution of lung tumor pathology review categories within decade of breast cancer diagnosis. This graph demonstrates that a similar percentage of cases were excluded from analysis from each decade of breast cancer diagnosis, except the 1990's, during when very few breast tumors in this study were diagnosed.

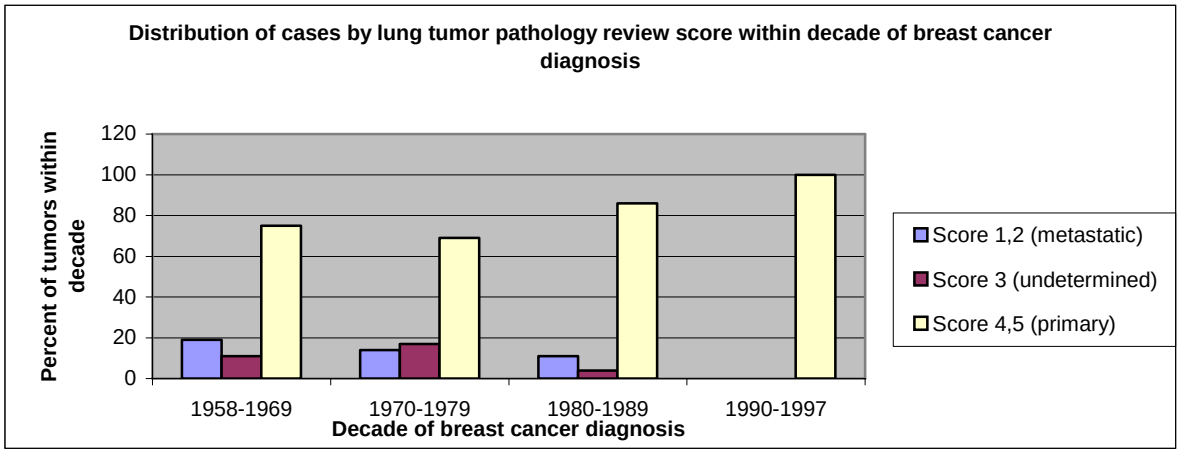


Figure 3. Distribution of lung tumor pathology review categories within decade of lung tumor diagnosis. No disparate percentage of cases were excluded from any particular decade of lung tumor diagnosis for the analysis.

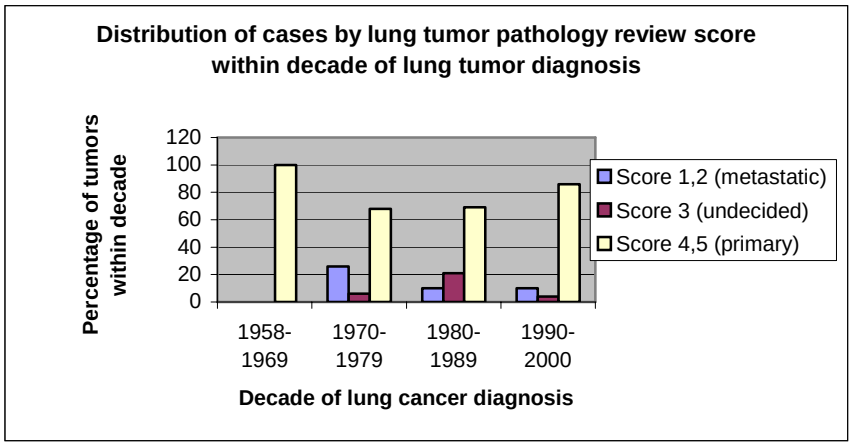


Figure 4. Distribution of cases by lung tumor pathology review categories across decades of lung tumor diagnosis. This graph demonstrates that more primary tumors were diagnosed in each progressive decade.

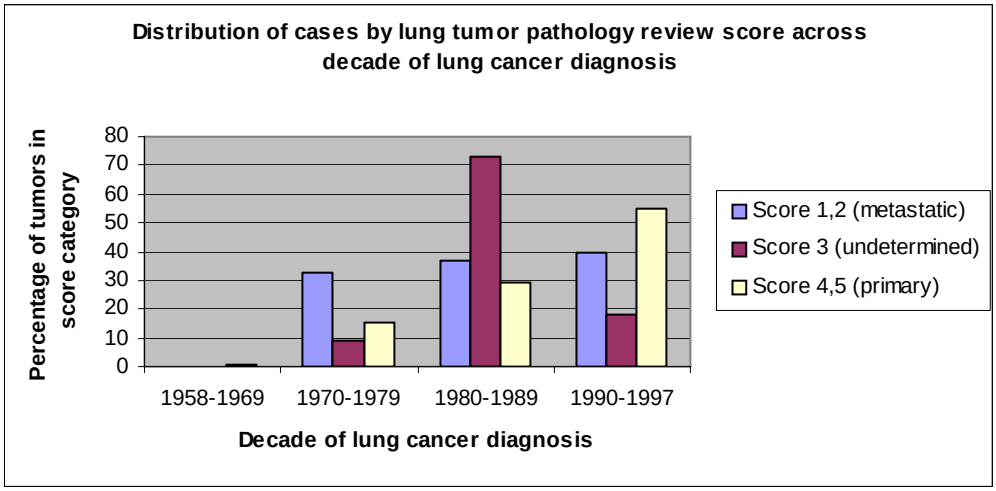
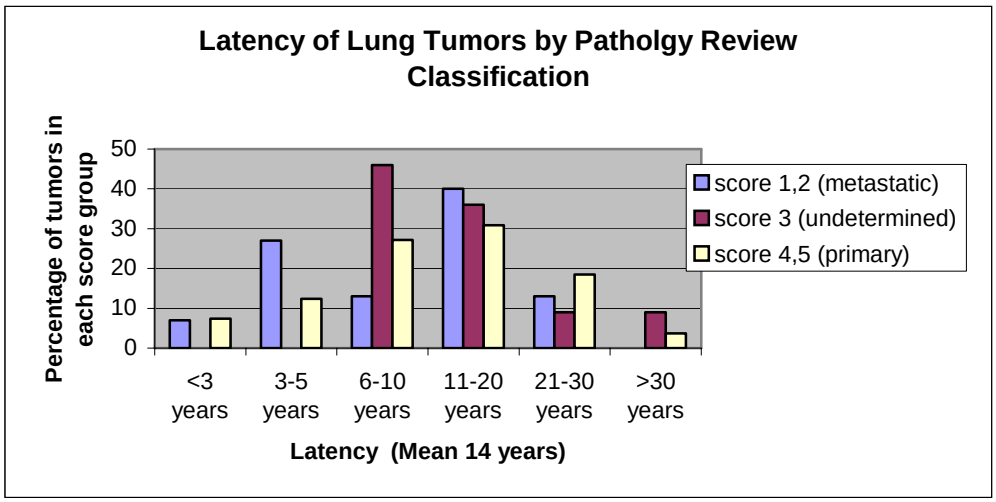


Figure 5. Distribution the pathology review groups by latency of lung tumor after breast cancer diagnosis. About half of the probable metastases had less than ten years latency, while about half of the undetermined tumors had greater than ten years of latency.



Task 1: To determine the mutational spectra of the p53 tumor suppressor gene in paired, non-synchronous breast and secondary lung tumors in women.

a. Extract DNA from slides of breast and lung tumor tissue from 220 case and 123 control tumors from the Swedish Cancer Registry.
b. Sequence DNA extracted from samples using PCR amplification and the Affymetrix microarray system, including 20% repeated for quality control.
c. Analysis of sequence data based on radiotherapy and smoking status.

The first task for this project was to extract DNA from breast and lung tumor tissue and to use extracted DNA in the Affymetrix microarray system to detect mutations in the p53 gene. We received 110 cases and 123 controls from our collaborators at the Karolinska Institute in Stockholm, Sweden. Samples were logged in to the laboratory tissue repository database system, given a numerical identifier,

and each slide was labeled with significant identifying information. DNA was extracted using a phenol-chloroform protocol. After extraction, samples were analyzed with a spectrophotometer to establish the concentration of DNA, normalized to 25ng/ul, and aliquoted to tubes for working stock and storage.

123 control breast tumors, 110 case lung tumors, and 110 case breast tumors were analyzed for *p53* mutations using the Affymetrix GeneChip system. 62% of lung tumors and 45% of breast tumors were amplified with a single PCR and analyzed by the GeneChip, while 38% and 55% respectively had to be amplified with a nested PCR protocol to generate enough PCR product to use in the GeneChip assay. 70% of samples were missing exon 4 data after hybridization to the GeneChip and 11% of lung tumors and 21% of breast tumors were missing data for other exons after the GeneChip hybridization. Individual exon sequencing was attempted to generate complete data for samples with missing exon data from the GeneChip, except for exon 4. The large size of exon 4 makes the GeneChip assay and sequencing analysis from paraffin-embedded, formalin-fixed tissues very challenging.

Validation was done for the nested protocol to verify that it would produce reliable data. 24 samples that had already been analyzed with the *p53* GeneChip assay using the Affymetrix primer mix and confirmed by sequencing were amplified using the nested primer protocol. All confirmed mutations were detected using the nested protocol, indicating that the protocol was able to accurately detect mutations that were identified by the original protocol. The nested strategy did result in more mutation scores, but all scores above 15 would be sequenced for mutation confirmation, so the nested strategy would not result in false positives (Table 4). To validate the technical skills of the researcher, a validation assay was done using samples from another study that had been evaluated using the *p53* GeneChip assay and confirmed by sequencing to be wild type or to have a *p53* mutation. The researcher was blinded to the status of the samples and produced results that were then compared to the original results. The 16 samples tested were confirmed, validating the researcher's technique. One sample did not amplify well and was missing exon 4 and 5 in the report produced from the GeneChip hybridization. The original result was wild type, so any inconsistency in the two missing exons would have been a false positive that would have been sequenced and invalidated (Table 5).

4 (4%) control breast tumors, 7 (7%) case breast tumors, and 20 (20%) lung tumors had a *p53* mutation and all were confirmed by repeat GeneChip analysis or sequencing with new PCR products (Tables 6 and 7). Transition mutations were most the common type in all tumors, comprising 100% of case breast tumor mutations and 75% of control breast tumor mutations. All mutations in breast tumors were located in exons 5-9. Missense mutations were the most common effect, comprising 86% of case breast and 50% of control breast tumor mutations. 86% of mutations in case breast tumors and 100% of mutations in control breast tumors were in the evolutionarily conserved region of *p53*. 43% and 29% of case breast tumor mutations were located in the DNA binding region and L2/L3 loop respectively (Table 6). In lung tumors, missense mutations were the most common effect of mutations (75% of mutations), the most common mutation location was the evolutionarily conserved region of the *p53* gene (60% of mutations), the most common change was A>G (26% of mutations), the most common exon with a mutation was exon 5 (33% of mutations), and transition mutations were the most common type of mutation (65% of mutations) (Table 7).

p53 mutations predict risk of secondary lung cancer: The odds ratio for risk of lung cancer with a *p53* mutation was 3.0 (CI 0.3-29.0)for samples with clinical data and 5.7 (CI 0.2-138.0) when adjusted for smoking and RT, but was not significant (Table 8). Chi-square analysis of the concordance of mutations in case breast and lung tumors found no significant association (Table 9).

Predictors of p53 mutations in secondary lung cancer: Chi-square analysis of the association of radiotherapy and *p53* mutations in lung tumors found no significant difference, but a trend toward an

increased risk of a *p53* mutation with no radiotherapy was observed (Table 10). Chi-square analysis of *p53* mutations in lung tumors and smoking status (yes or no) found no significant association between mutations and positive smoking status, but a trend toward an increased risk of a *p53* mutation with positive smoking status was observed (Table 11).

Table 4. Description of nested PCR protocol validation results, demonstrating the ability to accurately reproduce results with the adjusted protocol.

ID#	GeneChip results from PCR with Affymetrix primers*	GeneChip results from PCR with nested primers
13797	5(568)/15	wt
13824	5(575)/16, **7(930)/25**	5(660)/23, **7(930)/26**
13876	wt	5(480)/22, 6(706)/15, 6(715)/15
13888	5(517)/15	wt
13922	**7(906)/35**	**7(906)/26**, 5(660)/23, 5(454)/19
13946	wt	wt
13808	**5(570)/30**	**5(570)/27**
13821	wt	7(969)/15
13833	**5(484)/37**	**5(484)/29**, 5(665)/17
13961	**5(564)/21**	**5(564)/29**, 5(570)/18, 5(655)/17
13885	**7(921)/33**	**7(921)/33**
13781	5(568)/16, **7(888)/29**	**7(888)/16**, 6(706)/15, 8(999)/16
13795	wt	wt
13796	wt	5(517)/15
13840	wt	wt
13850	wt	5(660)/23
13866	**8(1045)/22**	5(562)/10, **8(1045)/23**
13875	wt	5(570)/17
13881	wt	wt
13889	5(492)/16,**6(824)/31**, 8(1066)/17, 8(1112)/20	**6(824)/32**
13897	wt	7(972)/15, 8(1077)/15, 10(1282)/15
13916	**9(1197)/33**	6(715)/16, **9(1197)/26**
13924	wt	5(475)/17
13947	**8(1072)/35**	**8(1072)/27**
13949	wt	wt

* wt- wild type; Results= Exon(GeneChip location number)/GeneChip score

Table 5. Description of researcher skill validation results for the *p53* GeneChip assay, demonstrating the ability of the researcher to reproduce previously verified results.

ID #	Original GeneChip Result	Repeat GeneChip Result
11500	wt	no exon 4 or 5, no scores >10
11502	codon 234 t>c	codon 234 t>c
11505	codon 278 c>t	codon 278 c>t
11506	wt	wt
11663	wt	wt
11668	codon 342 c>t	codon 342 c>t
11719	wt	wt
13606	codon 238 t>c	codon 238 t>c
13981	codon 342 c>t	codon 342 c>t
14087	codon 220 t>a	codon 220 t>a
14189	wt	wt
14355	wt	wt
14519	wt	wt
14541	codon 182 c>t	codon 182 c>t
14547	codon 238 t>g	codon 238 t>g
15871	codon 145 t>c	codon 145 t>c
16864	wt	wt

Table 6. Description of *p53* GeneChip mutation analysis results for breast tumors from cases and controls.

	Control		All case breast[1]		Scores 4, 5 case breast[2]		Score 3 case breast[3]		Score 1,2 case breast[4]	
p53	n=99	%	n=100	%	n=73	%	n=9	%	n=16	%
wild type	95	96	93	93	69	95	8	73	14	88
any mutation	4	4	7	7	4	5.5	1	27	2	12
Mutation type	n	%	n	%	n	%	n	%	n	%
ga	0	0	0	0	0	0	0	0	0	0
ag	1	25	4	40	3	75	1	100	0	0
ct	0	0	1	10	0	0	0	0	1	50
gc	2	50	1	10	0	0	0	0	1	50
tc	1	25	1	10	1	25	0	0	0	0
ac	0	0	0	0	0	0	0	0	0	0
ca	0	0	0	0	0	0	0	0	0	0
gt	0	0	0	0	0	0	0	0	0	0
transition	3	75	7	100	4	100	1	100	2	100
transversion	1	25	0	0	0	0	0	0	0	0
Mutation effect	n	%	n	%	n	%	n	%	n	%
missense	2	50	6	86	3	75	1	100	2	100
nonsense	1	25	1	14	1	25	0	0	0	0
silent	1	25	0	0	0	0	0	0	0	0
Mutation region	n	%	n	%	n	%	n	%	n	%
L2L3 loop	0	0	2	29	0	0	0	0	0	0
DNA binding	0	0	3	43	2	50	0	0	1	50
ECR	4	100	6	86	3	75	1	100	2	100
Exon	n	%	n	%	n	%	n	%	n	%
exon 5-9	4	100	7	100	4	100	1	100	2	100
5	3	75	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	3	43	1	25	0	0	2	100
8	1	25	3	43	2	50	1	100	0	0
9	0	0	1	14	1	25	0	0	0	0

1-Breast tumors from all cases with tissue available identified in the Swedish Cancer Registry. 2- Breast tumors from only cases with lung tumors with scores of 4 or 5 in the pathology review. 3- Breast tumors from only cases with lung tumors with a score of 3 in the pathology review. 4- Breast tumors from only cases with lung tumors with scores of 1 or 2 in the pathology review.

Table 7. Description of *p53* GeneChip mutation analysis results from case lung tumors.

	All case lung[1]		Scores 4, 5 prime case lung[2]		Score 3 case lung[3]		Score 1,2 case lung[4]	
p53	n=98	%	n=73	%	n=11	%	n=14	%
wild type	78	80	58	79	9	82	11	79
any mutation	20	20	15	21	2	18	3	21
Mutation type	n	%	n	%	n	%	n	%
ga	2	10	2	13	0	0	0	0
ag	5	25	4	27	0	0	1	33
ct	4	20	3	20	0	0	1	33
gc	3	15	1	6.7	1	50	1	33
tc	3	15	3	20	0	0	0	0
ac	1	5	0	0	1	50	0	0
ca	1	5	1	6.7	0	0	0	0
gt	1	5	1	6.7	0	0	0	0
transition	13	65	8	53	2	100	3	100
transversion	7	35	7	47	0	0	0	0
Mutation effect	n	%	n	%	n	%	n	%
missense	15	75	12	80	1	50	2	67
nonsense	3	15	2	13	0	0	1	33
silent	2	10	1	6.7	1	50	0	0
Mutation region	n	%	n	%	n	%	n	%
L2L3 loop	8	40	5	33	1	50	2	67
DNA binding	3	15	3	20	0	0	0	0
ECR	12	60	8	53	1	50	3	100
Exon	n	%	n	%	n	%	n	%
exon 5-9	20	100	15	100	2	100	3	100
5	7	35	5	33	0	0	2	67
6	3	15	2	13	1	50	0	0
7	4	20	3	20	0	0	1	33
8	5	25	4	27	1	50	0	0
9	1	1	1	6.7	0	0	0	0

1- All cases with tissue available identified in the Swedish Cancer Registry. 2- Only cases with scores of 4 or 5 in the pathology review. 3- Only cases with a score of 3 in the pathology review. 4- Only cases with scores of 1 or 2 in the pathology review.

Table 8. Regression analysis of *p53* mutations and lung cancer risk in breast tumors of cases versus controls. *p53* mutations in breast tumors may be associated with increased risk of lung cancer.

	Control	Case	OR	95% CI
p53 mutation all samples with assay data	4	7	1.3	0.3-6.0
p53 mutation only samples with clinical data	1	7	3.0	0.3-29.0
p53 mutation with clinical data and adjusted for smoking and RT			5.7	0.2-138.0

Table 9. Chi-square analysis of concordance of *p53* mutations in the breast and lung tumors of cases. No concordance of tumors with mutations is apparent.

	Lung	
Breast	Negative	Positive
Negative	49 (80)	12 (20)
Positive	2 (50)	2 (50)

p=0.2

Table 10. Chi-square analysis of p53 mutations in case lung tumors and exposure to radiotherapy. p53 mutations appear to be predictive of decreased risk of lung cancer with radiotherapy exposure.

	p53 mutation	
	negative	positive
No RT	16 (67)	8 (33)
RT and lung tumor less than 10 yrs after breast cancer diagnosis	15 (79)	4 (21)
RT and contralateral lung tumor greater than 10 yrs after breast cancer diagnosis	7 (100)	0 (0)
RT and ipsilateral lung tumor greater than 10 yrs after breast cancer diagnosis	20 (87)	3 (13)

p=0.2

Table 11. Chi-square analysis of *p53* mutations in case lung tumors and smoking status. Smoking may be associated with *p53* mutations in lung tumors.

	p53 mutation	
Smoking	Negative	Positive
Yes	36 (73)	13 (27)
No	16 (89)	2 (11)

p=0.2

Task 2: To determine ERA expression in paired, non-synchronous breast and secondary lung tumors in women and to establish primary tumor status of lung tumor tissue.

a. Perform immunohistochemical assays using ERA antibodies on breast and lung tumor tissue slides from 110 cases and 123 controls from the Swedish Cancer Registry.
b. Analysis of slide staining.

The second task for this project is to use immunohistochemistry to determine the ER alpha status of the breast and lung tumors and to establish the primary tumor status of the lung samples. 5 micron slides obtained from the tumor blocks were stained for ERA expression using ERA monoclonal antibody F-10 from Santa Cruz Biotechnology (Santa Cruz, CA), which recognizes the carboxy terminus of the receptor protein. Citrate acid buffer was used for antigen retrieval, the antibody was used at 1:25 dilution for 1 hour at room temperature, followed by the StriAveGen Multilink Kit, staining with diaminobenzidine chromogen solution (DAB), and counterstaining with hematoxylin (all reagents from Biogenex; San Ramon, CA). Slides were examined by microscope for the presence of ERA staining and compared to the positive and negative control slides for each experiment. Determination of positive or

negative expression status was made using the Allred scoring system, where numerical scores from 0-5 for proportion of tumor stained and 0-3 for intensity of staining are added for a final score; two or higher is considered positive for ER expression [82]. All slides were double read by a pathologist and 20% were repeated for quality control.

110 breast tumors and 105 lung tumors from cases and 117 control breast tumors were stained for ERA. 81% of control breast tumors and 74% of case breast tumors had an Allred score of 2 or greater and were considered positive (Table 12). 10% of lung tumors were positive for ERA (Table 10). Of the 103 cases that had ERA data for the breast and lung tumor, 8% were concordant for ERA positive status and 26% were concordant for ERA negative status. Overall, most concordance was in ERA negative cases. Most ERA negative concordance was in cases with lung tumors scoring 5 (primary tumors) in the pathology review and most ERA positive concordance was in cases with lung tumors scoring 1-3 (metastatic or undecided) (Table 14).

A similar percentage of cases concordant for ERA positive and ERA negative were excluded from analysis based on pathology review. In breast tumors, 74% of excluded tumors were ERA positive and 26% were ERA negative. In lung tumors, 30% of excluded tumors were ERA positive and 70% were ERA negative. The ratio of ERA positive to negative tumors is the same in excluded and included breast tumors. The percentage of ERA positive lung tumors that were excluded is larger than the percentage of included ERA positive lung tumors, probably because ERA positive status in a lung tumor suggests the tumor is likely metastatic.

ERA predicts lung cancer risk after breast cancer: Distribution of Allred scores for all tumors is shown in Tables 12. Figure 7 shows the distribution of Allred scores in cases with pathology scores of 4 or 5. (All analysis was done only on cases with lung tumors that scored 4 or 5 in the pathology review.) Conditional regression analysis indicated that positive ERA status in breast tumors is associated with decreased risk of lung cancer after breast cancer (OR 0.42, CI 0.26-0.89) (Table 15 and Figure 8). Breakdown of the samples into Allred scores categories of 2-5 and 6-8 shows the effect is confined to Allred scores 6-8 (OR 0.06, CI 0.008-0.5)(Table 15). Regression analysis using individual Allred score categories was not possible due to small sample size.

Analysis of ERA positive status and tumor latency or age at breast cancer diagnosis shows that the decreased risk of secondary lung cancer with positive ERA status in breast tumors is not associated with age at breast cancer diagnosis (also a substitute for menopausal status) but is associated with protection from lung tumors that arise less than ten years after breast cancer (OR 0.08, CI 0.01-0.7) (Table 16). Analysis of an interaction between latency and Allred score was not possible due to small sample size, but the protective effect related to Allred score in cases with lung tumor pathology review scores of 4 or 5 appears to be confined to an Allred scores of 8 and 7 (Table 17). Further subgroup analysis of interactions between ERA status and smoking or radiotherapy was not possible due to small sample size.

Distribution of cases and controls by age greater or less than 50 (surrogate for menopausal status) and ERA status compared to radiotherapy and smoking exposure is found in Table 18. Distribution of ERA status in breast and lung tumors of cases with lung tumor pathology review scores of 4 or 5 found no significant concordance (Table 19).

Table 8. Description of ERA IHC results from breast tumors of cases and controls.

ER alpha	Control		All case breast[1]		Scores 4 and 5 prime case breast[2]		Score 3 case breast[3]		Score 1,2 case breast[4]	
Status	n=117	%	n=110	%	n=82	%	n=10	%	n=16	%
positive	95	81	81	74	60	73	7	70	12	75
negative	22	19	29	26	22	27	3	30	4	25
Allred score										
0	22	19	29	26	22	27	3	30	4	25
2	0	0	8	7	6	7	0	0	2	13
3	1	1	4	4	0	0	2	20	2	13
4	6	5	8	7	6	7	2	20	0	0
5	12	10	12	11	10	12	1	10	1	6
6	14	12	11	10	8	10	1	10	1	6
7	18	15	15	14	13	16	1	10	0	0
8	44	38	23	21	17	21	0	0	6	37

1-Breast tumors from all cases with tissue available identified in the Swedish Cancer Registry. 2- Breast tumors from only cases with lung tumors with scores of 4 or 5 in the pathology review. 3- Breast tumors from only cases with lung tumors with a score of 3 in the pathology review. 4- Breast tumors from only cases with lung tumors with scores of 1 or 2 in the pathology review.

Table 13. Description of ERA IHC results from case lung tumors.

ER alpha	All case lung[1]		Scores 4, 5 prime case lung[2]		Score 3 case lung[3]		Score 1,2 case lung[4]	
Status	n=105	%	n=81	%	n=11	%	n=13	%
positive	10	10	2	2	3	27	5	38
negative	95	90	79	98	8	73	8	62
Allred score								
0	95	90	79	98	8	73	8	62
2	4	4	1	1	1	9	2	14
3	0	0	0	0	0	0	0	0
4	3	3	0	0	2	18	1	8
5	1	1	0	0	0	0	1	8
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	2	2	1	1	0	0	1	8

1- All cases with tissue available identified in the Swedish Cancer Registry. 2- Only cases with scores of 4 or 5 in the pathology review. 3- Only cases with a score of 3 in the pathology review. 4- Only cases with scores of 1 or 2 in the pathology review.

Table 14. Description of ERA status by pathology review score for case breast and lung tumors and description of concordance between case breast and lung tumors. A pathology review score was assigned to each lung tumor during the review conducted at KI by two pathologists, who reached a consensus for each lung tumor. The breast tumors are listed according to the score that their paired lung tumor received.

n=103	Breast				Lung				Concordance			
Pathology review score	ER +	%	ER -	%	ER +	%	ER -	%	ER +	%	ER -	%
1	2	2	0	0	2	2	0	0	2	2	0	0
2	8	8	3	3	3	3	8	8	3	3	3	3
3	7	7	3	3	2	2	8	8	2	2	3	3
4	9	9	3	3	1	1	11	11	0	0	2	2
5	49	48	19	18	1	1	67	65	1	1	19	18

Figure 7. Distribution of Allred scores in case and control breast tumors. The largest difference between cases and controls occurred in the Allred score category of 8.

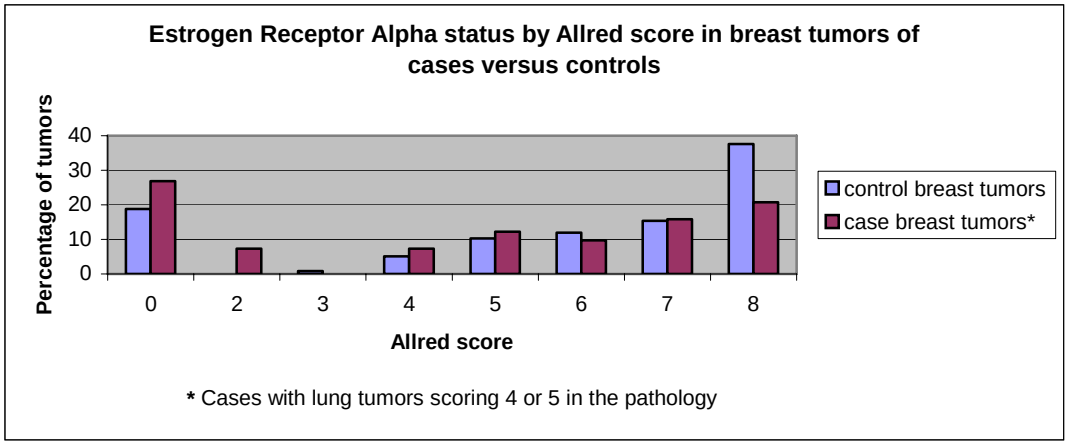


Table 15. Regression analysis of ERA status or Allred score for ERA status in case breast tumors versus control breast tumors. ERA positive status in breast tumors leads to a decreased risk of lung cancer. This protective effect appears to be confined to the higher Allred scores.

	n		crude analysis		adjusted*	
Factor	case	control	OR	95% CI	OR	95% CI
ERA	64	69	0.42	0.2-1.03	0.26	0.07-0.89
Allred 2-5	25	13	1.6	0.5-5.6	1.4	0.2-9.0
Allred 6-8	39	56	0.19	0.06-0.6	0.06	0.008-0.5

*adjusted for smoking and radiotherapy
n= number of positive samples

Figure 8. Odds ratios for the Allred score groups of 2-5 and 6-8 compared to <2. The OR for Allred score group 6-8 is significantly lower than the OR for the Allred score group 2-5.

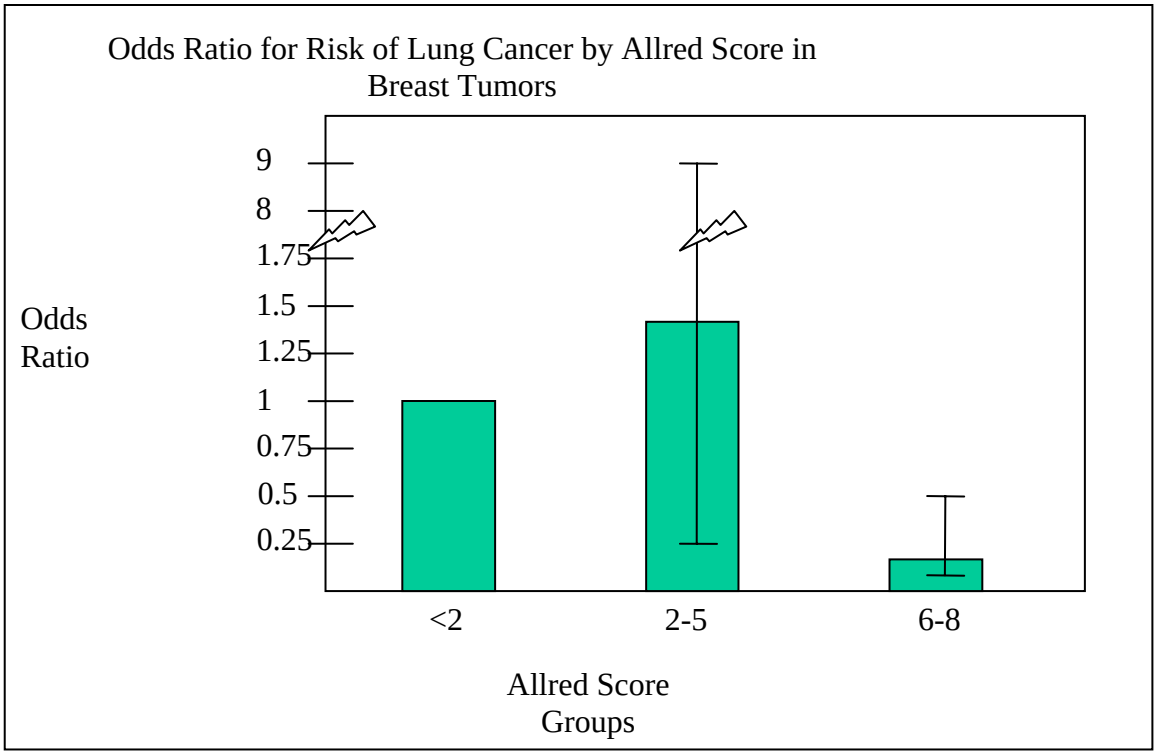


Table 16. Regression analysis of ERA status in breast tumors of cases versus controls and lung tumor latency or age at breast cancer diagnosis. The protective effect of ERA positive status in breast tumors appears to be confined to lung tumors arising less than ten years after breast cancer diagnosis.

	OR	95% CI
<10 yr latency*	0.08	0.01-0.7
>10 yr latency	1.4	0.6-3.4
age <50 at breast cancer diagnosis	1.2	0.4-4
age >50 at breast cancer diagnosis	0.4	0.1-1.2

* In controls, latency is survival time matched to the paired case lung tumor.

Table 17. Description of ERA by Allred score in case and control breast tumors grouped by latency. The protective effect of ERA positive status in breast tumors from lung tumors arising less than ten years after the diagnosis of breast cancer appears to be confined to the Allred score group of 8.

	Latency			
	<10 yr		>10 yr	
Allred score	Control	Case	Control	Case
0	1 (3)	20 (25)	16 (35)	12 (28)
2	0	4 (11)	0	4 (9)
3	0	1 (3)	0	0
4	2 (5)	0	1 (2)	5 (11)
5	3 (8)	3 (8)	5 (11)	8 (17)
6	4 (11)	2 (5)	7 (15)	6 (13)
7	8 (20)	6 (16)	1 (2)	6 (13)
8	20 (53)	12 (32)	16 (35)	4 (9)

Table 18. Description of the distribution of exposure to smoking or radiotherapy in cases and controls in groups defined by age at breast cancer diagnosis and ERA status. Small sample numbers do not permit further subgroup analysis by regression.

	age <50				age >50			
	ER+		ER-		ER+		ER-	
	case	control	case	control	case	control	case	control
Smoking								
yes	13 (76)	6 (43)	5 (71)	2 (50)	20 (69)	8 (31)	7(78)	2 (40)
no	4 (24)	8 (57)	2 (29)	2 (50)	9 (31)	18 (69)	2 (22)	3 (60)
Radiotherapy								
yes	8 (47)	4 (27)	4 (67)	2 (100)	4 (15)	2 (6)	3 (25)	1 (25)
no	9 (53)	11 (73)	2 (33)	0	23 (85)	33 (94)	9 (75)	3 (75)

Table 19. The concordance of ERA status in breast and lung tumors of cases. No concordance is apparent.

	Lung ER status	
Breast ER status	positive	negative
positive	3 (5)	63 (95)
negative	1 (4)	23 (96)

p=1.0

Task 3: To determine methylation status of *p16* and *Ecad* in breast and secondary lung tumors in women.

a. Perform PCR-based methylation assays on DNA extracted from 110 case and 123 control Swedish Cancer Registry samples, including 20% repeats for quality control.
b. Analysis of methylation patterns between breast and secondary lung tumors.

The third task for this project is to determine the methylation status of genes in breast and lung tumor tissue. DNA extracted from tumor slides is subjected to bisulfite treatment, which results in the deamination of unmethylated cytosines. Deaminated cytosines become uracils, which are recognized as thymines by the Taq polymerase used in PCR. PCR is then performed using primers that differentiate between the methylated sequences and the unmethylated sequences, where thymines are substituted for cytosines.

106 case breast tumors, 99 case lung tumors, and 122 control breast tumors had the 2ug of DNA required for bisulfite modification. Quality control analysis for DNA was done prior to bisulfite modification by

conducting PCR for *Methionine Synthase* (MS). The MS assay was first validated using a set of pedigree DNA; results were compared to the pedigree to verify that they were consistent with Harvey-Weinberg equilibrium. Frequency of MS genotype AA was 75%, 73%, and 68% in control breast tumors, case lung tumors, and case breast tumors respectively. Frequency for MS genotype AG was 20%, 26%, and 29% in control breast tumors, case lung tumors, and case breast tumors respectively. Frequency for MS genotype GG was 6%, 1%, and 3% in control breast tumors, case lung tumors, and case breast tumors respectively. These results are in agreement with published values [83-85].

A real-time PCR assay was used to detect methylation on the Taqman 7900 (Applied Biosystems). Modified DNA is used as template with specific primers and probes corresponding to the methylated sequence. Primers and probes were designed by Applied Biosystems Assay-by-Design product. The next quality control step occurred after bisulfite modification and determined the integrity of the bisulfite treated DNA using a real-time PCR assay for *b-actin*, a region that does not have methylated sites. DNA integrity was confirmed for 87% of case breast tumors, 82% of case lung tumors, and 86% of case breast tumors. All samples were tested for methylation of *p16* and *Ecad* regardless of *b-actin* status and one *b-actin* negative sample was positive for *p16* methylation. This sample was recorded as positive for methylation and not repeated in the bisulfite modification process.

Output from the real-time PCR report was compared to the positive control report for each PCR run to determine the methylation status of each sample. If a sample had a curve similar to the positive control and crossed the amplification threshold assigned by the analysis software, it was considered methylated in the promoter region of the particular gene being analyzed. *Ecad* methylation was detected in 10% of lung tumors and no breast tumors. *p16* methylation was detected in 15% of lung tumors, 2% of control breast tumors, and 4% of case breast tumors. 2% of control breast tumors, 4% of case breast tumors and 21% of lung tumors had at least one gene methylated and 4% of lung tumors had both genes methylated (Tables 20 and 21).

Predictors of methylation status in secondary lung tumors: 6 (15%) samples with *p16* methylation were from smokers and 1 (7%) was from a nonsmoker. Chi-square analysis of methylation and smoking status in lung tumors with pathology review scores of 4 or 5 found a non-significant increase in *p16* methylation in tumors from smokers compared to tumors from non-smokers (Table 22). Chi-square analysis of methylation in lung tumors and radiotherapy exposure found a significantly increased risk of *p16* or *Ecad* methylation in women who had an ipsilateral lung tumor that was diagnosed greater than ten years after radiotherapy for breast cancer, compared to women with breast cancer who did not receive radiotherapy or developed lung tumors with different latency or location. When the results were analyzed for *p16* only, the difference became statistically insignificant (Table 23). The frequency of *Ecad* methylation was too low to allow for analysis of the gene alone. When radiotherapy exposure was categorized into four groups instead of two, there was a non-significant trend for any methylation or *p16* methylation alone toward an increased risk of methylation in ipsilateral lung tumors that were diagnosed greater than ten years after radiotherapy (Table 24 and Figure 9).

Table 20. Description of methylation results in case and control breast tumors.

	Controls		**All case breast[1]**		**Scores 4, 5 prime case breast[2]**		**Score 3 case breast[3]**		**Score 1,2 case breast[4]**	
Methylation	n=105	%	n=92	%	n=70	%	n=8	%	n=12	%
Any	2	2	4	4	4	6	0	0	0	0
p16	2	2	4	4	4	6	0	0	0	0
Ecad	0	0	0	0	0	0	0	0	0	0
Both	0	0	0	0	0	0	0	0	0	0

1-Breast tumors from all cases with tissue available identified in the Swedish Cancer Registry. 2- Breast tumors from only cases with lung tumors with scores of 4 or 5 in the pathology review. 3- Breast tumors from only cases with lung tumors with a score of 3 in the pathology review. 4- Breast tumors from only cases with lung tumors with scores of 1 or 2 in the pathology review.

Table 21. Description of methylation results in case lung tumors.

	All case lung[1]		Scores 4, 5 prime case lung[2]		Score 3 case lung[3]		Score 1,2 case lung[4]	
Methylation	n= 80	%	n=61	%	n=9	%	n=11	%
Any	17	21	14	23	3	33	0	0
p16	12	15	9	15	3	33	0	0
Ecad	8	10	7	11	1	11	0	0
Both	3	4	2	3	1	11	0	0

1- All cases with tissue available identified in the Swedish Cancer Registry. 2- Only cases with scores of 4 or 5 in the pathology review. 3- Only cases with a score of 3 in the pathology review. 4- Only cases with scores of 1 or 2 in the pathology review.

Table 22. Chi-square analysis of *p16* methylation in case lung tumors. There is a nonsignificant trend toward methylation of p16 in lung tumors of cases and smoking.

	p16 methylation	
Smoking	Negative	Positive
Yes	34 (85)	6 (15)
No	14 (93)	1 (7)

p=0.7

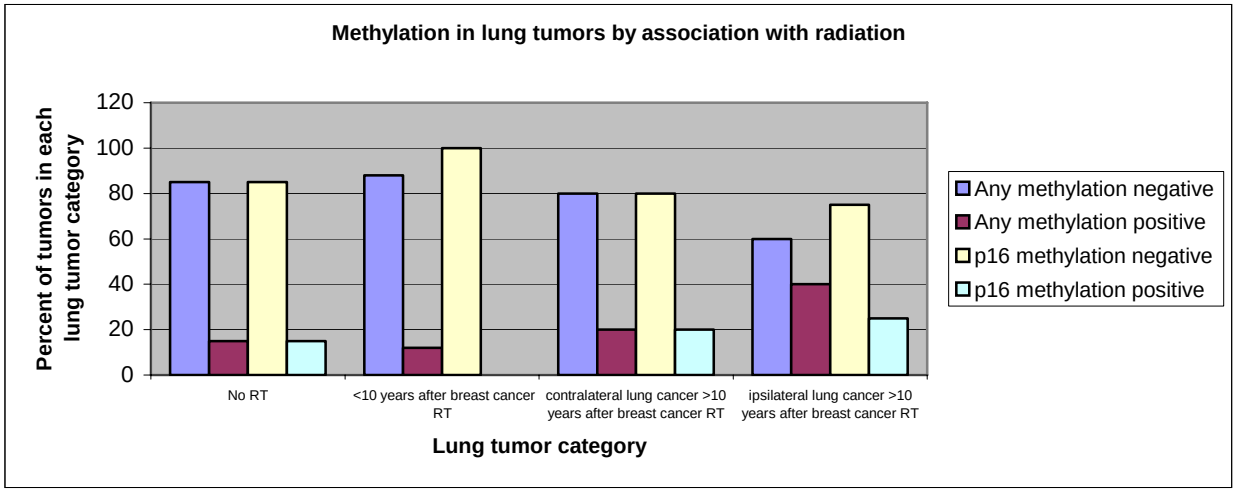
Table 23. Chi-square analysis of methylation in case lung tumors and radiotherapy exposure. A significant association between *p16* or *Ecad* methylation in lung tumors and exposure to RT with a subsequent ipsilateral lung tumor diagnosed greater than ten years after breast cancer diagnosis. When *p16* is analyzed alone, the difference becomes nonsignficant.

	Any methylation		*p16* methylation	
	Negative	Positive	Negative	Positive
RT ipsilateral lung tumor >10 yrs after breast cancer	12 (60)	8 (40)	15 (75)	5 (25)
Other (no RT, RT and lung tumor <10 yrs after breast tumor, RT contralateral lung tumor >10 years after breast tumor	35 (85)	6 (15)	37 (90)	4 (10)
	Chi square=5.0 p=0.03		Chi square=2.0 p=0.14	

Table 24. Chi-square analysis of methylation in case lung tumors and detailed radiotherapy exposure. The association seen for any methylation loses significance when radiation exposure and lung tumor latency and location are further stratified, however, a trend is observed suggesting that methylation is associated with radiation-induced lung tumors.

	Any methylation		*p16* methylation	
	Negative	Positive	Negative	Positive
No RT	17 (85)	3 (15)	17 (85)	3 (15)
RT with lung cancer diagnosis less than 10 years after breast cancer	14 (88)	2 (12)	16 (100)	0 (0)
RT and contralateral lung cancer greater than 10 years after breast cancer	4 (80)	1 (20)	4 (80)	1 (20)
RT and ipsilateral lung cancer greater than 10 years after breast cancer	12 (60)	8 (40)	15 (75)	5 (25)
	p=0.2		p=0.2	

Figure 9. These results demonstrate an increase in any methylation and *p16* methylation with lung tumors likely to be radiation-induced.



Key Research Accomplishments and Training (2003-2005)

- 343 tumors received, recorded, and labeled
- 343 tumors extracted for DNA
- 297 tumors analyzed for p53 mutations by Affymetrix Gene Chip
- 235 tumors stained for estrogen receptor alpha
- 277 tumors examined for *p16* and *Ecad* methylation
- Attendance at the Johns Hopkins School of Public Health Graduate Summer Institute in Epidemiology and Biostatistics, Baltimore, MD; 2003, 2004.
- Attendance at the National Cancer Institute's course in Principles and Practice of Cancer Control and Prevention, Bethesda, MD; 2003
- Attendance at weekly Tumor Biology Program Journal Club and Tumor Biology Data meeting seminars
- Attendance at bimonthly lab meetings and weekly student meeting with Dr. Shields

Reportable Outcomes

-Abstract presented at Lombardi Cancer Center Research Fair 2003-2005, Georgetown University, Washington, DC
-Data presented at the Tumor Biology Program Data Meeting 2003-2005, Georgetown University, Washington, DC
-Abstract presented at AACR Annual Meetings 2004 and 2006
-Abstract presented at 2005 Era of Hope Meeting, Philadelphia, PA
-Abstract presented at 2005 AACR Lung Pathogenesis Meeting, San Diego, CA
-Scholar-in-Training Award, 2005 AACR Lung Pathogenesis Meeting, San Diego, CA
- Publication: Tennis M, Krishnan S, Bonner M, Ambrosone CB, Vena JE, Moysich K, Swede H, McCann S, Hall P, Shields PG, Freudenheim JL. *p53* mutation analysis in breast tumors by DNA microarray. Cancer Epidemiol Biomarkers Prev 2006 Jan;15(1):80-5.
-Abstract presented at 2006 ASPO Annual Meeting, Bethesda, MD
-Cancer Prevention Fellowship, 2006 ASPO Annual Meeting
-AFLAC Scholar-in-Training Award, 2006 AACR Annual Meeting, Washington, DC

Conclusions

This population-based study examined the risk factors for secondary lung cancer among women with primary breast cancer residing in the Stockholm, Sweden area since 1958. Secondary lung cancer is a well-documented breast cancer survivorship issue. We looked for biomarkers in breast and lung tumors by investigating *p53* mutations, hypermethylation of *p16* and *Ecad* promoter regions, and ERA status. We first confirmed the pathologic classification of lung tumors, finding that about 25% of the lung tumors might be metastases misclassified as primary tumors. We then demonstrated that there is a reduced risk of lung cancer in women with breast cancers that have the highest expression of ERA and that this protective effect seems to be confined to tumors that are diagnosed less than ten years after breast cancer diagnosis. We have also demonstrated that there is an association between radiotherapy exposure and methylation of *p16* or *Ecad* in the lung tumors that occurred after breast cancer on the same side as the breast cancer. Our data suggests that there is an increased risk of lung cancer with a *p53* mutation in the original breast tumor and that there may be an association between the absence of *p53* mutations in the lung tumors and radiotherapy exposure, though this was not significant. The data was also consistent

with the hypothesis that smoking is associated with *p53* mutations in lung tumors, but the number of subjects was small and the finding was not statistically significant.

Pathology confirmation: Of fundamental importance to any study examining second cancers is the establishment of the primary status of the second tumors. This issue is also important for diagnosis and treatment of tumors in a clinical setting, as different approaches would be used for a metastasis in the lung versus a tumor originating in the lung. Most studies of second cancers rely on registry information and are unable to perform additional review to confirm that second tumors are primary and not metastases. Lung tumors after breast cancer can be especially challenging because lung adenocarcinomas can have similar morphology to breast adenocarcinomas. The lung is a common site of metastasis from breast cancer, with over 10-20% of patients hospitalized with breast cancer developing a metastasis in the lung. In an examination of isolated pulmonary nodules in women with a history of breast cancer, 39% of patients were treated for a primary lung tumor after a diagnostic workup [86]. 44% of patients were presumed to have metastatic breast cancer and tissue diagnosis was not pursued. Evaluation of factors often used as indicators of the histology of a pulmonary nodule after breast cancer, such as age, size of breast tumor, number of nodules, or axillary lymph node involvement, did not reliably exclude primary lung tumors, suggesting that there may have been some primary lung tumors in that study that were presumed to be metastatic breast tumors.

Algorithms developed to classify tumors of unknown origin facilitate correct clinical diagnosis, but in a retrospective study, when tissue or medical records are unavailable, researchers must rely on the recorded diagnoses [87]. These registries may be subject to recording bias, diagnoses based on outdated technology or knowledge, or basic recording errors. We had access to medical records and tissue blocks through the Swedish Cancer Registry, effectively giving us the ability to review the tissues as the original diagnosing physicians did. The study of second cancers is greatly enhanced by this unprecedented access, however, the identification of primary lung tumors was still challenging.

Unlike any previous study, we had pathology samples for our subjects and were able to incorporate fresh IHC stains (H&E, ERA, and TTF-1) into an algorithm for classification of lung tumors. First, the H&E slides of the breast and lung tumors from each patient were compared for morphologic similarities or differences. TTF-1 stains 65-95% of lung adenocarcinomas and is highly specific for lung and thyroid tissue; thus it has been suggested as a useful clinical marker to differentiate between primary and metastatic tumors in the lung [87-90]. A positive TTF-1 stain in our study was cause for classification of the lung tumor as primary. ERA status alone did not classify lung tumors as primary or metastatic, however, breast metastases are more likely to maintain the ERA status of the primary tumor, so an ERA positive lung tumor from a patient with an ERA positive breast tumor was considered a probable metastasis or undecided in the absence of decisive morphology [91-94].

There are no prior studies that have confirmed the pathological diagnosis of secondary lung cancer. In the two published studies of lung cancer following breast cancer that were able to do some evaluation of the status of lung tumors, both relied on review of medical records and not pathology samples for confirmation [14;20]. The study by Ford et al. excluded 4% of potential cases because they were unable to rule them out as metastases and excluded 8% of potential cases because the breast or lung carcinoma was misclassified in the registry. By examining the actual pathology samples, our study used a more rigorous evaluation of lung tumors and excluded 25% of potential cases from the analysis because we were unable to confidently classify them as primary tumors. By working with two pathologists to reach agreement on the status of lung tumors, we established a stronger case for each lung tumor. The major reason for excluding samples from our study after the pathology review was the similarity of morphology between the breast and lung tumors without a positive TTF-1 stain of the lung tumor. The original

pathologic diagnosis may have been based more on misleading clinical factors (presentation, latency, etc.) rather than histologic factors.

In previously published studies, misclassification of lung tumors could have the effect of underestimating the risk of lung cancer after breast cancer. The cohort study using the Swedish Cancer Registry conducted prior to this study would have contained up to 25% metastases that would have been randomly distributed between contralateral and ipsilateral tumors, reducing the association of lung cancer risk with ipsilateral location [95]. The follow up to this study that found an increased risk of lung cancer with radiotherapy could have been subject to additional underestimation of risk because the possible metastases would have been randomly distributed between the no radiotherapy and radiotherapy groups [21]. In studies using the Connecticut Tumor Registry and SEER, some found an increased risk of lung cancer with exposure to radiotherapy, however, these studies may also be subject to up to 25% misclassification of lung tumors. The resulting underestimation of risk may be contributing to the different levels of risk detected by different studies [8;9;13;96-99]. In the study conducted at MD Anderson Cancer Center, a review of medical records was done that caused 4% of cases to be excluded for lack of evidence supporting primary status of the lung. Using only a medical record review, however, could result in some metastases still being classified as primary, which may account for their lack of association between radiotherapy and lung cancer risk [20]. Two studies conducted using the National Surgical Adjuvant Breast and Bowel Project excluded 17% and 3% of lung tumors as possible metastases after review of medical records. The first study found an increased risk for lung cancer with radiation that disappeared when the possible metastases were added into the analysis. The second study found no association between radiotherapy and lung cancer risk. The results from these studies support the suggestion that underestimation of lung cancer risk after breast cancer may occur with misclassification of metastases [14].

This study does not indicate that misclassification occurs strictly by decade of diagnosis. After the pathology review, 11 tumors were classified as undetermined and 16 as probable metastases, excluding them from the analysis. These exclusions were distributed among decades of breast cancer diagnosis, with the greatest number of probable metastases found in the earliest decade and decreasing with later decades. This may be due to changes in diagnostic criteria since 1958, resulting in more metastatic tumors misidentified as primary in earlier decades. When latency of the different pathology categories was examined, all of the undetermined tumors had latencies of greater than six years and about half had latencies greater than ten years. Based on the expectation that secondary lung tumors arise later than metastases, it is more likely that this group contains primary tumors compared to the probable metastasis group, in which half of the lung tumors had latencies of less than ten years.

Currently, adenocarcinoma is the most frequent type of lung cancer, followed by squamous cell carcinoma [100]. In our study, adenocarcinoma was the most common type of lung cancer when all cases were included, but when only tumors classified as definitely or probably primary were included, it was the second most common. Squamous cell carcinoma was the second most common when all cases were included and the first when only definitely or probable primary cases were included. Small cell carcinoma was third in both groups of cases. All of the cases excluded from our analysis were adenocarcinomas or another form of non-small cell lung cancer. Adenocarcinoma is the histological type most likely to be misclassified because of the similar morphology of adenocarcinomas from different tissues, so these are the hardest to confirm as primary. The clearer differences between squamous or small cell carcinoma and breast adenocarcinoma made those tumors more easily confirmable as primary.

Smoking, radiotherapy, and risk of secondary lung cancer: In any study of lung cancer risk, smoking plays an important role. Studies of second primary lung cancers that are limited by a lack of smoking

information and cannot provide a complete picture of lung cancer risk. In our study, 68 (62%) cases were smokers, of which 9 (13%) were identified through next-of-kin interviews, and 23 (19%) controls were smokers, of which 4 (17%) were identified through next of kin interviews. Our study was strengthened by access to medical records for all patients and by the use of a validated method to collect smoking information from next-of-kin interviews. With smoking information, our study was better able to assess lung cancer risk within the context of exposure. Radiotherapy is also emerging as an exposure that may increase the risk of lung cancer after breast cancer treatment [9;13;101]. Our study had access to radiotherapy information from medical records and the ability to calculate doses to each side of the lung, providing more detailed information on radiotherapy than any previous registry study. Unfortunately, dose information was not available in time for the analysis of this study, but will be included in future analyses. Crude odds ratio analysis confirmed results from previous studies in this population indicating increased risk of lung cancer after breast cancer with exposure to radiotherapy for breast cancer or for smoking [21].

Distribution of ERA staining: In previous studies, about 70% of breast tumors and anywhere from 0% to 65% of lung tumors were found to express ERA [35;37]. In our study, 81% of control breast tumors, 74% of all case breast tumors, and 10% of all case lung tumors expressed ERA. When cases were excluded as undetermined or probable metastases, expression of ERA in lung tumors dropped to 2%, while expression in breast tumors remained the same. Though a small percentage of primary lung tumors do express ERA, it is more likely that an ERA expressing lung tumor is a metastasis [37;87]. Comparing our results to studies of ERA expression in lung tumors in the literature is challenging. There is a wide variability in recorded expression that could be due to study sample size, antibody choice, antigen retrieval techniques, or quantification of positive expression. Studies of estrogen receptors and exposure to radiation in cell lines suggest that radiation inhibits estrogen-induced cell growth [102;103]. Radiotherapy for breast cancer may then prevent the growth of ERA positive lung tumors, resulting in a larger number of subsequent lung tumors with ERA negative status. If a study uses patients that have received different types of treatment for breast cancer, ERA status of lung tumors in the populations may be affected by the different treatments. 65% of cases in this study with confirmed primary lung tumors received radiotherapy, which could result in a largely ERA negative lung tumor group.

Very little concordance for ERA status between breast and lung tumors of cases was observed. Most of the concordance occurred for ERA negative cases with lung tumors that were considered definitely primary in the pathology review. Concordance for ERA positive cases was much lower and most were undetermined or probable metastases in the pathology review, excluding them from analysis. The discordance could be due to the influence of radiotherapy in 65% of the cases with a primary lung tumor. Lack of concordance of ERA suggests that the breast and lung tumors from cases do not have a common hormonal etiology. No previous studies of lung cancer after breast cancer have analyzed the concordance of ERA status between tumors, so additional studies should be done to validate these results. Care must be taken to standardize the analysis of ERA expression in lung tumors in order to produce accurate results.

Breast cancer ERA staining is a secondary lung cancer risk factor: The Allred scoring system was used to classify ERA staining in breast tumors. 81% of control tumors and 74% of case breast tumors had an Allred score of 2 or greater and were considered positive. This is the first report of ERA staining as a marker of risk in for secondary lung cancer in women with breast cancer. When ERA status was analyzed as a marker for risk of lung cancer, positive ERA status in the breast tumor was significantly associated with a decreased risk of lung cancer (OR 0.26, CI 0.07-0.89). Analysis of ERA status based on Allred score found that the protective effect of ERA positive status was confined to the Allred score group of 6-8 (OR 0.06, CI 0.008-0.5). Further regression analysis of each individual Allred score was not possible due to small sample size.

Positive ERA status in the breast tumor was significantly associated with an increased risk of lung cancer occurring less than ten years after the original breast cancer (OR 0.08, CI 0.01-0.7). ERA positive status may be associated with an increased risk of lung cancer with a greater than ten year latency, however, because the confidence intervals for the OR include one, the possibility that ERA positive status is also protective for lung tumors with short latency cannot be ruled out (OR 1.4, CI 0.6-3.4). However, the fact that the greatest effect was in women with lung tumors of less than ten-year latency indicates that this is not likely solely a survival effect. ERA status was not found to be associated with age at breast cancer diagnosis and risk of lung cancer (surrogate for menopausal status). Analysis of the interaction between latency and Allred score was not possible due to small sample size, but the frequency table shows that the protective effect observed for latency less than ten years occurs mostly for Allred score 8. The small sample numbers in many categories leaves open the possibility that additional samples would change the distribution of Allred scores. If the addition of samples reinforced the observation from this analysis, however, the fact that high expression of ERA leads to reduced risk suggests a biological effect.

The complicated role of ERA in breast cancer, combined with the intention of the examination of ERA in this study to be only hypothesis generating, makes interpretation of these results challenging. An Allred score of 8 is the strongest possible expression detected through IHC. Breast cancers that strongly express ERA may be part of an environment that is resistant to future cancers compared to ERA negative tumors, as women with ERA positive breast tumors have a better overall prognosis. This would be supported by a finding that ERA positive status is also protective for tumors with a greater than ten year latency. Women with ERA negative breast tumors may be predisposed aggressive cancers, like lung cancer. Analysis of a sample set of breast cancer patients with ERA data for risk of any second cancer might find an increase in tumors with particularly bad prognosis associated with ERA negative breast cancer. There may be a classification bias if diagnosing physicians for some reason were more inclined to record lung nodules in women with ERA positive breast tumors as metastases. If physicians regarded lung tumors in women with strongly ERA positive breast tumors as even more likely to be metastases, the excess of tumors with an Allred score of 8 might be expected. If, however, it were simple ERA positive status that led to the assumption of metastases, this misclassification would presumably be present across the spectrum of Allred scores. Unfortunately, there were not enough samples to adequately assess this possibility. ERA positive status in a breast tumor is not known to be, however, a marker of increased risk for pulmonary metastases, so it is unlikely that this misclassification exists [93]. If the analysis of ERA had been conducted using all cases in this study, including cases classified as possible metastases, a protective effect of ERA positive breast tumors could have been attributed to the erroneous presence of breast cancer patients with metastatic lung tumors in the case group who would be more likely to be ERA negative. In our analysis, however, only cases with a confirmed primary lung tumor were included, making it unlikely that misclassification could account for the observed protective effect of ERA positive breast tumors.

It has been suggested that an unequal distribution of Allred scores may be due to technical issues. Two studies published in 2005 suggested that expression of ERA in breast cancer was essentially bimodal [104;105;105]. Both studies used the Allred scoring system and found the majority of tumors had Allred scores of 0 or 7 and 8. Criticism of this conclusion suggested that the distribution of Allred scores in ERA staining of breast cancer is highly sensitive to antigen retrieval technique and tissue fixation [106]. Antigen retrieval and tissue fixation problems would, however, lead to broader distribution of Allred scores among samples, as potentially strong ERA expressing tissues might appear weakly expressing with suboptimal fixation or antigen retrieval techniques. If case and control breast tumors in this study were somehow treated differently in fixation, this effect might be seen; however, cases and controls were matched by decade of diagnosis, which should reduce the possibility of different techniques being used.

As expected, smoking appears to increase the risk of lung cancer regardless of ERA status of breast tumors or age at breast cancer diagnosis. Analysis of risk of secondary lung cancer due to the interaction between age at breast cancer diagnosis, smoking or radiotherapy, and ERA status was not possible due to small numbers, however, the data suggest that there may be some interaction. A sample size of at least 224 would be necessary to detect an OR of 2.0 with 90% power for all of these interactions. Women diagnosed with breast cancer at age less than 50 may have an increased risk of lung cancer with smoking if their breast tumor is ERA positive compared to women older than 50 at breast cancer diagnosis (76% younger than 50 versus 69% older than 50). Radiotherapy may increase the risk of lung cancer in this study, particularly in women with ERA positive breast tumors diagnosed at age less than 50 compared to women diagnosed at age greater than 50 (47% younger than 50 versus 15% greater than 50). Women with aggressive tumors may be more likely to develop secondary lung cancer and if they are diagnosed at an age younger than 50, may have a longer time to live in which to be affected by exposures and subsequently develop lung cancer.

Distribution of hypermethylation of p16 and Ecad: Gene promoter hypermethylation is emerging as an important mechanism for the gene silencing that plays a role in tumorigenesis. Results of published studies provide a wide range of comparison. Methylation of *Ecad* has been published at 18-87% in lung tumors and 18-80% in breast tumors [52;53;107-110]. Methylation of *p16* has been published at 17-79% for lung tumors and 3-75% for breast tumors [51;55;109;111-117]. The large variation, which could result from different histology, tumor stage, or method of detection, makes it difficult to compare results between studies. *Ecad* methylation was detected in 10% of lung tumors and no breast tumors. *p16* methylation was detected in 15% of lung tumors, 2% of control breast tumors, and 4% of case breast tumors. Our study found low rates of methylation for all tissues, which was unexpected, but all validation of our assays indicates that the results are correct. The *p16* methylation rate for lung tumors is close to rates from previous studies, while the rate for breast tumors matches the lowest published rate. Methylation for *Ecad* in lung tumors is lower than published rates, and no *Ecad* methylation was detected in breast tumors.

Low methylation rates could be due to technical issues or particular characteristics, as yet unknown, of this population. Methylation analysis can be technically challenging due to the harsh bisulfite treatment that occurs before PCR. The samples in this study were formalin-fixed, paraffin-embedded tumors that were collected as far back as 1958. Quality of DNA and the amount of DNA available from extractions was a significant concern. Distribution of samples by success of modification (assessed by *b-actin* analysis) suggested that samples from earlier decades were less likely to have sufficient high-quality DNA after bisulfite modification. This may introduce a bias that affects methylation rates. Loss of Ecad expression is associated with aggressive breast cancer, but since the women in this study survived long enough to get a second cancer, it is less likely that they had loss of Ecad expression [118]. It is possible that this population will have lower *Ecad* methylation rates than more diverse breast cancer patient populations. It was unexpected, however, that no samples had *Ecad* methylation and evaluation of a larger number of cases will be required to make any conclusions regarding this data.

Exposures may predict methylation in secondary lung tumors: An association between methylation of *Ecad* or *p16* and exposure to radiotherapy was observed in women with ipsilateral lung tumors diagnosed less than ten years after breast cancer. Chi-square analysis of methylation in lung tumors and radiotherapy exposure found a significantly increased risk of *p16* or *Ecad* methylation in women who have an ipsilateral lung tumor that was diagnosed greater than ten years after radiotherapy for breast cancer, compared to women with breast cancer who did not receive radiotherapy or developed lung tumors with different latency or location ($p=0.03$). A significant result was only observed for the comparison between women with radiotherapy and ipsilateral tumors diagnosed less than ten years after breast cancer versus all other radiotherapy exposure or lung tumor location. When radiotherapy and lung

cancer location were divided into four categories, the association was no longer significant but the trend toward methylation in radiotherapy influenced tumors was apparent. The addition of more genes to the methylation analysis might increase the frequency of methylation and lead to significant results from the detailed analysis. The same loss of significance occurred when *p16* was analyzed alone, which was unexpected because there were so few *Ecad* methylated samples contributing to the overall effect. This further demonstrates the instability of an analysis with so few samples, but the addition of samples from the larger study will likely rectify this problem.

A previous cohort study that used the Swedish Cancer Registry to investigate lung cancer risk after breast cancer found an increased risk of ipsilateral lung cancer greater than ten years after diagnosis of breast cancer. The laterality and latency of the lung tumors in this study suggested that these lung tumors were radiation induced. Radiation dose information was not yet available from medical records for our study, which limited the analysis of radiation exposure. A dose-relationship for methylation and radiation exposure was observed in a study of plutonium factory workers [78-80]. There is evidence that methylation of *p16* in lung tumors is associated with smoking (25-60% of tumors) in a dose-dependent manner [76;119-121]. Smoking data is available for 75% of our cases, but dose information is limited. We detected no significant increase in *p16* methylation in lung tumors of smokers. Again, this finding would be improved with the addition of more cases to the study; based on the frequency of methylation of lung tumors from smokers in our study, at least 224 cases would be required to detect an OR of 2.0 at 90% power. Smoking could also be examined for an interaction with radiotherapy exposure with the addition of more cases.

Some studies of *p16* methylation in lung tumors have found higher rates of methylation in squamous cell lung tumors compared to other histologies and for tumors in smokers compared to nonsmokers [121;122]. There were too few *p16* methylated tumors to perform analysis on the data, but of the nine *p16* methylated lung tumors included in this study, four were adenocarcinoma, two were adenocarcinoma/squamous cell carcinoma mixed, two were squamous cell carcinoma, and one was small cell carcinoma. This distribution does not agree with published studies, but additional samples may add to the power necessary to confidently assess the association of methylation with histology.

Distribution of p53 mutations: We identified *p53* mutations in 21% of lung tumors classified as primary by the pathology review. This rate is lower than the 50% mutation rate recorded in the IARC database for lung tumors. Our most common mutation effect was missense (80%) and it was similar to the IARC database (75%) [23]. Nonsense and silent mutations occurred slightly more often in our study than in the IARC database (13% and 7% versus 8% and 4%). Our most common alteration was AT>GC (47%), which occurred less frequently in the IARC database (11%). The most common alteration in the IARC database was GC>TA (30%), which only accounted for 14% of our mutations. Transitions make up 27% and transversions 51% of the mutations in the IARC database, while they comprised 53% and 47% respectively in our study. Deletions make up 9% of the IARC *p53* mutation database and, while the Affymetrix GeneChip system can detect single base pair deletions, we did not identify any in our study. Sequencing was done for deletion scores of seven or higher because they were infrequent and the deletion scores were consistently lower than mutations on the score report. It is possible that the GeneChip was very sensitive for the tissue in this study and that very low scores were given to deletions, causing them to be missed by our score cutoff. We did not identify any insertions because the Affymetrix GeneChip system does not detect insertions, but this would lead to the exclusion of only 2% of *p53* mutations, according to the IARC database. In breast tumors that were included in the final analysis (both cases and controls), the *p53* mutation rate was 4% for control breast tumors and 7% for case breast tumors for our study. This rate for breast tumors is considerably lower than the IARC breast tumor database rate of 26% [23]. 56% and 26% of IARC breast tumor mutations were transitions and transversions, compared to 75% and 25% in control breast tumors and 100% and 0% in case breast tumors in our study. In IARC

compared to our study, there were 67% versus 62% missense mutations, 8% versus 25% nonsense mutations, and 5% versus 12% silent mutations. The most common alteration in the IARC database was GC>AT (42%), which did not occur in our study. AT>GC comprised 75% of our mutations but only 11% of mutations in IARC.

There is no obvious technical reason for the low rates of *p53* mutations in this study. Sequencing was done for all scores above 15, the cutoff score that was validated with other study sets tested in our lab. Other published studies have confirmed mutations with lower scores, however, so it is possible that the GeneChip has different sensitivities in different sample sets. This could have resulted in samples with low mutation scores that were missed by the cutoff of 15. Validation of user skills with a set of samples of known mutation status confirmed that technique did not affect the study results. Validation of the nested PCR protocol demonstrated that altering the standard PCR protocol did not result in false negatives, thus not causing the low mutation rate, and allowed more samples to be included in the assay. Low mutation rates may also be due to unknown unique qualities of this population, since *p53* mutations in subjects with breast cancer and a secondary lung tumor have not been thoroughly studied. *p53* mutations are associated with more aggressive breast cancers, especially aggressive ERA negative breast cancers, so the fact that the women in this study were 75% ERA positive and survived their breast cancers long enough to get a second cancer suggests that there may be a lower rate of *p53* mutations than in the general breast cancer population [24]. The lack of *Ecad* methylation in breast tumors supports this as well. A study of primary breast carcinomas found that *p53* mutations were significantly associated with *Ecad* methylation, suggesting that there may be a correlation between low rates of *p53* mutations and *Ecad* methylation [108]. The use of low-quality DNA could also be a technical issue that may lower the *p53* mutation rate in our study. The amplification rate of exon 4 in the multiplex PCR was low, a result of paraffin-embedded, formalin-fixed tissues. The distribution by decade of diagnosis for samples that failed GeneChip analysis suggests that, at least for lung tumors, older samples are more likely to fail.

p53 breast cancer mutations may predict secondary lung cancer risk: Regression analysis of *p53* mutations was challenging due to low sample size and low frequency of mutations in all tumor types. Our data suggests that there is an increased risk of lung cancer with a breast cancer *p53* mutation, which increases when adjusted for radiotherapy and smoking. These results are not significant, however, as they are based on only four mutations in controls and seven in cases. A sample size of over 400 would be needed to detect an OR of 2.0 with 90% power at the frequency of *p53* mutations in breast tumors observed in our study.

Exposures may predict p53 mutations in secondary lung tumors: Chi-square analysis of *p53* mutations in lung tumors and exposure to radiotherapy or smoking was not significant. The data for *p53* mutations and radiotherapy has a trend toward more frequent mutations in women who were not exposed to radiotherapy. This observation was unexpected, as some studies have found *p53* mutations associated with radon exposure and radiation has been associated with DNA damage and genomic instability [123]. It may be that the low number of mutations in our cases is confounding the results. To detect an OR of 2.0 with 90% power for *p53* mutations and radiotherapy at the frequency of mutations observed in our study, at least 246 samples would be needed. The data for *p53* mutations and smoking status suggest that *p53* mutations in lung tumors are associated with smoking. While these data are not significant, they agree with previous studies [29]. To replicate these findings with 90% power and an OR of 2.0, the study size would need to be at least 159 cases.

There may be an interaction with radiation in smokers that would increase their chances of having a *p53* mutation in their lung tumors, although this was not supported by our data. Four of the smokers with a *p53* mutation had the expected GC>TA transversion mutation in their lung tumor that has been shown to be more frequent in smokers compared to never-smokers [29]. In the other smokers with *p53* mutations,

five had AT>GC mutations and three had GA>CT mutations. It is likely that the small number of mutations detected is not sufficient to adequately assess the distribution of mutation types. The concordance analysis of *p53* mutations in the breast and lung tumors of cases had only two mutations in each positive or negative breast tumor category, making the analysis unreliable and leaving open the possibility that concordance could exist when more cases are included in the study. There may be a correlation between *p53* mutations and *p16* methylation in lung tumors of women with exposure to smoking and/or radiation. A study published by Jarmalaite et al. found a correlation between *p53* mutations and *p16* methylation in large cell carcinomas. In our study, two of the nine lung tumors with *p16* methylation also had *p53* mutations; however, one was an adenocarcinoma and one was a small cell carcinoma.

Study strengths: This study is the first to examine biomarkers of risk in women with breast cancer and a second primary lung tumor. Conducting this study through the Swedish Cancer Registry provided access to data and pathology materials rarely available to studies of second cancer conducted in the United States. No published study to date has used pathology materials in the analysis of risk of lung cancer after breast cancer. We were able to review lung tumors and matched breast tumors from cases and to confirm the primary status of 77% of the lung tumors. The use of a robust DNA extraction protocol allowed the inclusion of samples from as far back as 1958, which increased the number of cases available for analysis. ERA staining data was near complete for this study, with information gathered on 95% of control breast tumors, 99% of case breast tumors, and 96% of case lung tumors.

Access to medical records allowed the gathering of detailed disease and exposure information. Smoking status is frequently missing in registry studies of second lung cancer, but our study had smoking information on 75% of cases. The use of a validated next-of-kin method for patients without smoking data in their medical records provided information on an additional 13% of cases. Breast cancer treatment information is also often missing from registry studies of lung cancer following breast cancer. We were able to gather data on radiotherapy treatment from medical records for 97% of cases, allowing analysis of the influence of exposure to radiation on secondary lung cancer risk. Use of samples from several decades allows the investigation of different radiation doses because of the diversity of breast cancer treatments given.

Study limitations: A possible selection bias occurred when cases were excluded due to lack of tissue. If clinical data were available for the excluded cases, it would be possible to compare the characteristics of cases excluded for lack of tissue to the included sample set to determine if a bias was introduced. The quality of samples spanning four decades of histologic technology is diverse and the methylation and *p53* mutation assays used in this study have fairly stringent DNA requirements. Technical issues relating to the use of paraffin embedded, formalin fixed samples may have confounded some of our results. Missing information on TTF-1 status for 50% of the tumors reviewed as possible metastases impeded our ability to comprehensively apply the algorithm developed by our pathologists.

Lack of sufficient quantity of DNA to perform the methylation analysis was a problem for less than 1% of control breast tumors, but was an obstacle for 4% of case breast tumors and 10% of case lung tumors. Poor DNA quality after bisulfite modification interfered with analysis of 13% of case breast tumors, 18% of case lung tumors, and 14% of control breast tumors. The lack of *Ecad* methylation in breast tumors eliminated the possibility of examining *Eca*d in breast tumors as a marker of lung cancer risk. The loss of methylation data significantly impacted all analyses of hypermethylation in breast tumors as a marker of lung cancer risk. *p53* mutation data was missing from 20% of control breast tumors and from 12% each of case breast and lung tumors, which probably related to the quality of the DNA. 70% of samples were missing *p53* mutation data for exon 4, likely due to formalin fixation, but mutations in exon 4 account for only 4% of *p53* mutations in breast cancer and 3% in lung cancer.

A clear limitation of this study was the small sample size, a problem that was magnified by the low frequency of the biomarkers assayed. We were unable to analyze data to investigate the hypothesis that *p53* mutations and hypermethylation of *p16* and *Ecad* are markers of an interaction between smoking and radiotherapy. With the addition of approximately 400 cases from the rest of Sweden as part of a large case control study, however, this interaction will be addressed with sufficient power. It is also possible that further analysis of a complete data set will point towards a genetic susceptibility in these women. An additional statistical limitation was the lack of clarification of the use of control variables and case-control matching characteristics in regression analysis. This data is available, but due to time restrictions, could not be included in this report.

Conclusions and perspectives: Radiotherapy provides a significant reduction in local recurrence in women with breast cancer. It should and will continue to be used as an integral part of the treatment plan for many breast cancer patients. This treatment, however, leads to an increased risk of lung cancer that should not be overshadowed by the success of radiotherapy. Surviving breast cancer only to be diagnosed with a possibly preventable lung tumor years later would be tragic. Biomarkers could identify subsets of women who may have an elevated risk of lung cancer if they choose to receive radiotherapy. Women who have breast tumors with good prognosis should be informed of their risk of treatment-related disease. Some women may choose not to accept the risks of radiotherapy and opt for mastectomy. Importantly, smokers may be convinced to quit with knowledge of additional risk information. Breast cancer patients will benefit from additional individualized risk information that, in the context of other clinical details, could alter the course of their treatment.

We identified ERA as a potential marker for decreased risk of lung cancer after breast cancer. We also described exposure to radiation as a predictor of hypermethylation in lung tumors. *p53* mutations in breast cancer may be a marker of increased lung cancer risk and smoking may be a predictor of *p53* mutations and hypermethylation in secondary lung tumors. Additional cases will allow detailed investigation of interactions between smoking and radiotherapy in subsets of women with different clinical characteristics. We also described possible misclassification of about 25% of lung tumors registered as primary tumors. TTF-1 data is still required for these samples to make definitive conclusions, but these results could have a significant impact on the interpretation of prior registry studies and could alter the design of future studies.

It will be valuable to continue this study with a larger sample size and to investigate chemotherapy as a risk factor for secondary lung cancer and the possible interaction with smoking. As additional samples accumulate, pathology review could identify more misclassified tumors that may be used in a study of women with breast cancer that metastasizes to the lung. The research presented in this dissertation is the first step in a unique collaboration utilizing the extensive epidemiologic information and pathology materials available through the Swedish Cancer Registry and the advanced technology of a molecular biology laboratory at Georgetown University. This study will provide new and important insight into the problem of treatment-related second cancer and possibly lead to treatment changes for some breast cancer patients.

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